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Biological Aspects of Metal-Binding

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BIOLOGICAL ASPECTS OF METAL-BINDING

MARVIN J. SEVEN, M.D.

FEDERATION PROCEEDINGS SEPTEMBER 1961
Supplement no. 10

PROCEEDINGS OF A CONFERENCE ON

BIOLOGICAL ASPECTS OF METAL-BINDING

Held at The Pennsylvania State University
University Park, Pennsylvania, September 6–9, 1960

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Edited by
L. Audrey Johnson, Philadelphia General Hospital
Marvin J. Seven, Graduate School, University of Pennsylvania

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*Roster of Participants**

ALBERT, ADRIEN	The Australian National University, Canberra, Australia
APOSHIAN, H. VASKEN	Vanderbilt University School of Medicine, Nashville, Tennessee
BORG, DONALD C.	Brookhaven National Laboratory, Upton, Long Island, New York
BOYLE, A. J.	Wayne State University, Detroit, Michigan
CALVIN, MELVIN	University of California, Berkeley
CATSCH, A.	Institute for Radiobiology, Karlsruhe, Germany
CHENOWETH, MAYNARD B.	The Dow Chemical Company, Midland, Michigan
CHRISTENSEN, HALVOR N.	University of Michigan Medical School, Ann Arbor
COTZIAS, GEORGE C.	Brookhaven National Laboratory, Upton, Long Island, New York
CURRAN, GEORGE L.	St. Louis University School of Medicine, St. Louis, Missouri
EICHHORN, GUNTHER L.	National Heart Institute, Bethesda, and Baltimore City Hospitals, Baltimore, Maryland
FOREMAN, HARRY	Los Alamos Scientific Laboratory, Los Alamos, New Mexico
FOYE, WILLIAM O.	Massachusetts College of Pharmacy, Boston
GOULD, R. GORDON	Stanford University School of Medicine, Palo Alto, California
GRYDER, J. W.	Johns Hopkins University, Baltimore, Maryland
GUSTAFSON, RICHARD L.	Clark University, Worcester, Massachusetts
HARDY, HARRIET L.	Massachusetts General Hospital, Boston
HASTINGS, A. BAIRD	Scripps Clinic and Research Foundation, La Jolla, California
KEHOE, ROBERT A.	University of Cincinnati College of Medicine, Cincinnati, Ohio
KROLL, HARRY	Montefiore Hospital, New York City
MALMSTRÖM, BO G.	University of Uppsala, Uppsala, Sweden
MARTELL, ARTHUR E.	Clark University, Worcester, Massachusetts
MARTIN, R. BRUCE	University of Virginia, Charlottesville
METZLER, DAVID E.	Iowa State University, Ames
NAJJAR, VICTOR A.	Vanderbilt School of Medicine, Nashville, Tennessee
OSER, BERNARD L.	Food and Drug Research Laboratories, Inc., New York City

* This roster includes participants presenting formal papers. A complete index of authors and discussants appears on page 263.

PEARSON, RALPH G.	Northwestern University, Evanston, Illinois
PERRY, H. MITCHELL, JR.	Washington University School of Medicine, St. Louis, Missouri
PETERS, HENRY A.	University of Wisconsin Medical School, Madison
PHILIPS, FREDERICK S.	Sloan-Kettering Institute for Cancer Research, New York City
PRICE, J. M.	University of Wisconsin School of Medicine, Madison
RAY, ROBERT D.	University of Illinois Research and Educational Hospitals, Chicago, Illinois
REILLEY, CHARLES N.	University of North Carolina, Chapel Hill
RUBIN, MARTIN	Georgetown University Medical School, Washington, D. C.
SCHEINBERG, I. HERBERT	Albert Einstein College of Medicine, New York City
SCHUBERT, JACK	Argonne National Laboratory, Argonne, Illinois
SCHWARZ, KLAUS	National Institutes of Health, Bethesda, Maryland
SNELL, ESMOND E.	University of California, Berkeley
VALLEE, BERT L.	Harvard Medical School, Boston, Massachusetts
WEINBERG, EUGENE D.	Indiana University, Bloomington
WESTERFELD, W. W.	State University of New York, Upstate Medical Center, Syracuse
WILLIAMS, R. J. P.	Wadham College, Oxford, England

Marvin J. Seven, M.D.

AN OBITUARY

Dr. Marvin J. Seven died suddenly at the age of 33, while in the process of editing the proceedings of this conference.

Dr. Seven was known to the participants of this conference as a vigorous proponent and investigator of chelation therapy, by which is meant the utilization of artificial ligands for the purposeful sequestration of certain elements within the human body. Although he had conducted investigations in this area, it is germane to recall that he had contributed considerably also to the dissemination and to the integration of the concepts which characterize this field. This he accomplished by means of the various scientific exhibits for which he was well known and, more importantly perhaps, by means of the symposium which he conceived and organized in Philadelphia in 1959. This symposium recognized the emergence of a new field in the biological sciences. It also defined this field as one which cuts across the boundaries of several scientific disciplines. The proceedings of that symposium were edited by Dr. Seven and were published under the well-known title *Metal-Binding in Medicine*. The popularity of this book and the subsequent emergence of several meetings based roughly on the pattern which was set by Dr. Seven bear ample testimony to his perceptiveness.

His high moral character and personal charm will be sadly missed by his many friends, who were looking forward to the full fruition of the excellent beginning of his scientific career.

George C. Cotzias
Medical Research Center
Brookhaven National Laboratory

Program Committee

HASTINGS, A. BAIRD, *Chairman*, Scripps Clinic and Research Foundation, La Jolla, California

CHENOWETH, MAYNARD B., The Dow Chemical Company, Midland, Michigan

COTZIAS, GEORGE C., Brookhaven National Laboratory, Upton, Long Island, New York

FOREMAN, HARRY, Los Alamos Scientific Laboratory, Los Alamos, New Mexico

MARTELL, ARTHUR E., Clark University, Worcester, Massachusetts

STAFF

WEISIGER, JAMES R., National Academy of Sciences, Washington, D. C.

Foreword

R. KEITH CANNAN

Division of Medical Sciences, National Academy of Sciences–National Research Council, Washington, D. C.

THIS FOREWORD will introduce the record of the proceedings of a conference sponsored by the Division of Medical Sciences, National Academy of Sciences–National Research Council and held at The Pennsylvania State University, 6–9 September 1960.

The Division is indebted to Dr. A. Baird Hastings who, as Chairman of the Conference and of its antecedent Planning Committee, contributed so much to give form and spirit to the Conference. In this, Dr. Hastings was ably supported by the chairmen of the several sessions and by the many contributors to the program. Particular acknowledgment should be made of the role of the late Dr. Marvin Seven in promoting the idea of holding a conference in this field and in contributing to its design and purpose. It is appropriate that there should be included in this volume a brief obituary of Dr. Seven prepared by Dr. George Cotzias.

The Conference became a reality only as a result of generous support provided by the Atomic Energy Commission and by the National Institutes of Health and as a result of the able and zestful staff work of Dr. James R. Weisiger. For the editorial disciplining of the fruits of the Conference into a form suitable for publication, the Division is indebted to Dr. L. Audrey Johnson.

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Introductory remarks

A. BAIRD HASTINGS

Scripps Clinic and Research Foundation, La Jolla, California

IT IS MY PLEASANT PRIVILEGE to welcome you to this Conference on the Biological Aspects of Metal-Binding. For a few days, we are to have the pleasant experience of exchanging information in an atmosphere of intimacy on this subject which is of common interest and of growing importance in many areas of the biological and medical sciences.

It was with reluctance that I agreed to serve as chairman because any one of you present is better equipped to do so than I. However, since each congregation of experts should have at least one attentive student, I have accepted my assigned role in that spirit—and I expect to learn a great deal.

I regret that we were unable to arrange for the presence here of all those who would have liked to be present. One of those who is unavoidably absent is Antoine Lavoisier. He had just reached the point in his experiments in which he was able to compile tables of the relative affinity of bases (including metals) for acids, when his career was abruptly terminated by "the sanguinary tyranny of the Monster Robespierre"—as his English contemporary expressed it.

In his absence, I venture to reproduce one of two dozen tables to be found in the 1789 edition of Lavoisier's *Elements of Chemistry*, listing in the order of their "affinity" (his term) the combinations of the "bases," as he called them, with citric acid. (I chose this from among the various other acids because of my own, now ancient, experience with the binding of calcium, strontium and magnesium with citrate.)

*Table of the Combinations of Citric Acid, with the Salifiable Bases, in the Order of Affinity**

Bases	Neutral Salts
Barytes	Citrate of Barytes
Lime	Citrate of Lime
Magnesia	Citrate of Magnesia
Potash	Citrate of Potash
Soda	Citrate of Soda
Oxyds of:	Citrate of Oxydated Metals
Zinc, Manganese, Iron, Lead, Cobalt, Copper, Arsenic, Mercury, Antimony, Silver, Gold, Platina	

* A. Lavoisier. *Elements of Chemistry*, 1789, p. 329. Other tables list: carbonic, tartarous, mallic, oxalic, succinic, gallic, lithic, lactic, sebacic and prussic acids.

Although ions and stability constants were still many years away, I could not but be impressed with the fact that his alkali citrates are in about the right order, insofar as I have information about them. I take it that he would be forgiven for not having listed the "affinities" of his metal citrates exactly in the order that we might expect today.

You will note also that Lavoisier's observations ranged widely through different acids of biological origin. One observation recorded in his discussion of prussic acid seemed to me especially relevant to our deliberations on this occasion.

Again, I quote from Lavoisier:

"It (Prussic Acid) combines with iron, to which it communicates a blue color, and is equally susceptible of entering into combination with most of the other metals."

Then he concludes:

"Although this acid combines with alkalies, earths, and metals, . . . it possesses only some of the properties we have been used to attribute to acids, ... But, as I have already observed, it is difficult to form a decided opinion upon the nature of this substance, until the subject has been farther elucidated by a greater number of experiments."

Yes, Monsieur Lavoisier, we have heard you loud and clear, and elucidation "by a greater number of experiments" is just what we have been doing, and on which we shall be exchanging information for the next few days.

In 1958 alone, *Chemical Abstracts* listed 110 references under Chelation, Chelatory agents, and Chelatometry. Of interest to us are papers on such subjects as:

Effect of chelation on the action of branching enzyme and phosphorylase.

——on phosphatase inhibition.

——on bacteriophage T-2 infectivity.

——enzyme formation in yeasts.

——on muscle contraction.

Effect of chelation of calcium of blood serum, on digitalis toxicity.

Chelation in chemotherapy.

Anticancer action and chelation of nucleic acids.

And chelation in relation to survival after irradiation.

Etc., etc.

Yes, there is no dearth of experimental work on *Biological Aspects of Metal-Binding*. What we hope to achieve from our present conference is better knowledge of guiding principles, ideas for improved methodology—and best of all, new experimental approaches to problems of biological and medical importance.

BACKGROUND OF CONFERENCE

This Conference on the Biological Aspects of Metal-Binding originated from the desires of a number of investigators, and Dr. Harry Kroll in particular, to have extended discussions of the subject by representatives from several disciplines in order to expand our knowledge of metal-binding agents, their mode of action and their application to problems in biology and medicine.

As you know, the Conference is sponsored by the Division of Medical Sciences of the National Academy of Sciences–National Research Council, and has received generous support from the Atomic Energy Commission and the National Institutes of Health. I am sure that you join me in expressing our indebtedness to all these organizations, and to Pennsylvania State College for making this meeting possible.

Dr. James Weisiger of the National Research Council has been principally responsible for the organization of the Conference (one might say he is a catalyst of Chelation). At his instigation, an ad hoc committee was appointed and met for two days to consider the subjects to be covered. This has resulted in the program upon which we are about to embark.

The program is to cover four main topics: *1*) Physical Chemistry of Chelation, *2*) Physiological Aspects of Metals, *3*) Pharmacology and Toxicology of Chelating Agents, and *4*) Applications of Chelating Agents in Medicine. Those named to present papers on one of these topics, or to open the discussion of the papers, have been active contributors to their field of special interest. It is to be hoped that they will emphasize the unsolved areas of their subjects, as well as those which are solved—and that all of the rest of you will consider yourselves on the program as active participants. The Proceedings of the Conference will be published as a supplement to *Federation Proceedings* and will be edited by Dr. Marvin Seven and Dr. Audrey Johnson. We are pleased, indeed, to have them do this.

Now, I should like to introduce to you the Chairmen of the Sections of the Conference.

Our Chairman for today's session is Dr. Arthur Martell, Professor of Chemistry, Clark University. He might appropriately be called Mr. Chelation, himself, and I am sure needs no introduction to anyone working on problems concerned with metal-binding.

Tomorrow's Chairman will be Dr. George Cotzias. He is Head of the Division of Physiology in the Department of Medicine at the Brookhaven National Laboratory. His interests have ranged from enzymes to irradiation and, for some time now, have been concentrated on the biological behavior of the alkaline earths and of manganese. He will be in charge of the session on the Physiological Aspects of Metals.

The Chairman of the third session will be Dr. Maynard Chenoweth, formerly at the University of Michigan—now Pharmacologist, Biochemical Research Laboratory, Dow Chemical Company. He is well known for his work on metal-binding in relation to pharmacological problems, and will preside over the session on the Pharmacology and Toxicology of Chelating Agents.

Dr. Harry Foreman will be Chairman of the fourth session. He is a member of the Research Staff of the Los Alamos Scientific Laboratory and has long been concerned with medical applications of metal-binding agents, such as EDTA.

Finally, our distinguished colleague from Berkeley, Professor Melvin Calvin will take charge of our last session. His research has run the gamut from physical and organic chemistry to all sorts of biological problems embracing the plant and animal world. I hope that, by then, he will be able to identify which questions we have answered—and which ones we have not.

If I were asked to formulate, in broad terms of reference, questions of biological import which might be considered at this Conference, I expect that I would first think of such questions as:

1. How much of each element is present?
2. How is it distributed in tissues and fluids?
3. In what chemical forms does it exist?
4. What reactions does it undergo?
5. If it is essential for normal life, how much is needed?
6. What are the upper and lower limits of concentration compatible with normal metabolic activity?

These are the questions that I have asked myself in the past when I have undertaken the study of such abundant constituents as sodium, potassium, calcium, magnesium, chlorine, phosphorus and sulfur. The large amounts of these elements present make such questions amenable to attack—though they are by no means all answered as yet.

However, when one considers the rarer constituents, such as iron, copper, zinc, molybdenum, manganese, cobalt and iodine, which are of primary concern to a conference such as this, one is confronted with quite a different set of problems. Where their biological roles have been identified, they are proven to be highly specific ones. This has led to the accumulation of a large body of information regarding the types of reactions into which these rarer body constituents enter.

And this, in turn, has led to quite a different list of questions. These include—

1. About what sort of binding of metals are we talking?
2. Is the binding reversible, as with calcium citrate—or irreversible as with ferro-protoporphyrin?
3. Is it the role of the ligand in controlling the metal ion concentration, or is it the combination

of the metal ion with the ligand which is biologically important?

4. What are the quantitative consequences of competition for the same metal by different ligands, and for the same ligand by different metals?

These and many others will doubtless be discussed at our Conference.

We biologists and medical scientists who are here will hope to gain information that will aid us to some extent in better understanding such subjects as: enzyme action, transport of materials across cell membranes, intermediary metabolism of food stuffs, and action of drugs, vitamins and hormones.

In the spirit of the beginning student, full of anticipation yet fearful that I am not prepared adequately for what lies ahead, I now turn the meeting over to our Chairman for today, Professor Arthur E. Martell.

I. PHYSICAL CHEMISTRY OF CHELATION

ARTHUR E. MARTELL, *Chairman*

Opening remarks by the chairman

ARTHUR E. MARTELL

THE FIELD of coordination chemistry has developed very rapidly during the past 10 to 15 years as a result of two major advances: *1*) the quantitative approach to coordination in solution, as evidenced by the large number of publications on stability constants that have appeared; and *2*) the development of the ligand field theory, which has helped considerably in our understanding of the coordinate bond for transition metals. The research activity associated with these two outstanding developments, plus a number of related advances such as metal titrations and biological functions of complexes, have resulted in the publication of about three thousand papers in recent years on metal complexes and chelates, and their applications.

An indication of the activity in these related fields can be found in the large number of monographs which have recently appeared. A number of these are listed in Table 1.

Many related books have been left off the list. For example: the applications of metal chelates to analytical chemistry have been described in books by Pribil and Flaschka. Also, much additional information can be found in a number of new books on inorganic chemistry such as *Advances in Inorganic Chemistry and Radiochemistry*, Volume I and II, edited by Emeleus and Sharpe, and Volume I of the series *Progress in Inorganic Chemistry*, edited by F. A. Cotton. Then, too, there are the valuable collections of papers describing the Proceedings of the International Conferences on Coordination Compounds held in Copenhagen, Amsterdam, Rome, and London.

The proliferation of publication in this field is being further maintained by a number of books now in preparation. I am awaiting with considerable interest the forthcoming volumes by Charles Reilley, F. J. C. Rosotti, F. Welcher, Orgel, Klixbull Jorgensen, M. Rubin, and many others.

The purpose of this session on physical chemistry of chelation is not to recite or catalog the existing chemical knowledge in the field, but rather: *1*) to attempt a conceptual understanding of the physical and chemical properties of coordination compounds as a function of their chemical constitution; and *2*) an interpretative description of some of the possible approaches to the problems involving metal ions in biological systems. The possibility of advances in this direction has been opened up as a result of the fact that it is now possible to attain some measure of control of metal ion activity by the use of chelating agents, as well as to modify their chemical properties by coordination with organic ligands of various types. By the use of this approach to coordination chemistry, the papers which follow are designed to describe the present state of knowledge of special reagents now available for metal ion control, as well as what is now known about the interactions of metal ions with the various types of groups found in biological systems.

TABLE 1. *Recent Books on Metal Complexes*

Date	Title	Author(s)
1941	Metal Ammine Formation in Aqueous Solution	J. Bjerrum
1952	The Chemistry of the Metal Chelate Compounds	A. E. Martell and M. Calvin
1954	Chemical Specificity and Biological Interactions	F. R. N. Gurd (Ed.)
1955	Die Complexometrische Titration	G. Schwarzenbach
1956	The Chemistry of the Coordination Compounds	J. Bailar (Ed.)
1958	Mechanisms of Inorganic Reactions	F. Basolo and R. G. Pearson
1958	EDTA	F. J. Welcher
1957	Stability Constants, Part I: Organic Ligands	J. Bjerrum, L. G. Sillén and G. Schwarzenbach
1958	Stability Constants, Part II: Inorganic Ligands	Same as above
1959	The Sequestration of Metals	R. L. Smith
1959	Organic Sequestering Agents	S. Chaberek and A. E. Martell
1960	Modern Coordination Chemistry	J. Lewis and R. G. Wilkins (Eds.)
1960	Chelation Phenomena	J. F. Frederick (Ed.)
1960	Metal-Binding in Medicine	M. J. Seven (Ed.)

Nature and properties of metal ions of biological interest and their coordination compounds

R. J. P. WILLIAMS
Inorganic Chemistry Department and Wadham College, Oxford, England

FORMATION OF COMPLEX IONS

a) Preferential Combination Between Ligand and Metal

ONE REMARKABLE PROPERTY of the cations of the periodic table is that in an aqueous solution the preference for one type of ligand as compared with another is a function of the cation. There are two consequences of this observation. Firstly, no simple electrostatic picture of the binding of cation and ligand can be satisfactory and, secondly, the combination of ligands and cations can become highly selective even if not specific. It is probably as well to take some inorganic systems as examples before discussing more complicated cases (Table 1). The thiocyanate ion presents two possible coordination centers, binding to a cation through either sulfur or nitrogen. Whereas in the majority of complexes of first row transition metal ions and in complexes of the elements in A subgroups of the periodic table the thiocyanate ion is bound through nitrogen, it is bound through sulfur in many complexes of the heavier B subgroup elements and also in the complexes of the later elements, after group VIIA, in the second and third rows of the transition metal series (9). Now, roughly the same division into these two groups of cations is maintained in the manner of combination with the nitrite ion (nitrogen binding or oxygen binding), with the thiosulfate ion (sulfur or oxygen binding), and in the orders of stability of the cation halide complexes which can be either

$$F^- > Cl^- > Br^- > I^- \quad \text{or} \quad I^- > Br^- > Cl^- > F^- \quad (1).$$

In extreme cases one cation can show two behaviors with one ligand. An interesting example is that of Co(III). Whereas the simple inorganic complexes of the cobaltic ion with thiocyanate and selenocyanate are nitrogen bound, the complexes of vitamin B_{12} are sulfur and selenium bound respectively. Here steric factors appear important.

This selectivity of reaction between ligand and cation can occur in simple competition reactions between two neutral molecules, e.g. ammonia and water, as well as in the competition between two sites of the same ligand. Thus ammines are formed by one group of cations and not by another just as some cations readily form fluoride complexes and others do not. These observations imply that water sometimes binds more firmly than ammonia or fluoride and sometimes less strongly. In some cases direct quantitative comparison of complex stabilities of a series of metal ions with different sets of ligands, as in Figure 1, is now possible. From these data we see that magnesium, calcium and manganese(II) prefer binding to oxygen-anion ligands over combination with simple neutral nitrogen-donor molecules, whereas nickel and copper make the opposite choice of ligand.

The selection of one ligand group rather than another is clearly dependent upon a number of factors. In the absence of differences in size of the different cations, a dependence on electronegativity is most obvious. The electronegativity effect is most important, for example, in the series of cations of Figure 1. As these cations are amongst the most common in biological systems, we have dealt with the comparative effect of their electronegativities on their properties in detail elsewhere (15). Now, cations outside this series differ in size as well as electronegativity. Gross steric factors therefore affect selective reaction. We will outline these factors before returning to show why steric factors are also important in controlling the stability of cations of similar size.

For example, in the reaction of cations with a hydroxy-acid the question arises as to whether the hydroxyl group will be bound or not. In this case there is a competition between three different kinds of donor, all oxygen atoms: *a*) that carrying the highest net charge—the carboxylate oxygen; *b*) that occupying the least volume in the coordination sphere—the water oxygen; *c*) the hydroxyl oxygen which is hindered from coordination by steric factors, the carbon chain to which it is attached, but is a better donor than oxygen of water. On formation of the hydroxy complex, there may be a considerable entropy gain (chelation) which will assist the displacement of water by the hydroxyl group. However, steric hindrance can reduce the possibility of complex formation with a group—although it is more able to donate electrons—below that of the less powerful donor. The hydroxyl group suffers most from steric hindrance as it is really an alkyl substituted water molecule. In this particular case of competition between

TABLE 1, (9). *Preferential Combinations Between Ligands and Metals*

Ligand	Cations Preferring Oxygen	Cations Preferring Sulfur	Cations Preferring Nitrogen
SCN^-		Hg(II), Ag(I), Pt(II), Rh(III), [Co(III)]	Ni(II), Co(II), Mn(II), Zn(II), Fe(II), MoO_2(II)
NO_2^-	Cr(III), Na(I)		Co(III), Cu(II), Ni(II), Ru(II)
$S_2O_3^{--}$	Ba(II)	Hg(II), Pd(II), Cu(I)	
SO_3^{--}	IA, IIA, IIIA cations	Hg(II), Pd(II), Pt(II)	
Sulfur containing amino acids	Mg(II), Ca(II), Mn(II), [Co(II)?]	Cu(I), Cu(II), Cd(II), Zn(II), Hg(II), Ag(I)	Mn(II), Co(II), Mg(II)

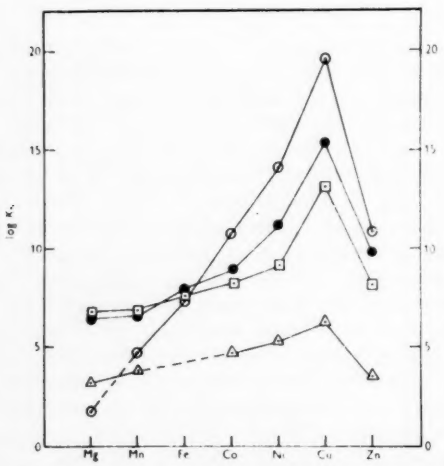


FIG. 1. The stability constants of some complexes of the divalent ions of the first transition metal series. ⊙, ethylenediamine complexes, log K_2 values. ⊡, salicylaldehyde complexes, log K_2 values. ●, glycine complexes, log K_2 values. △, oxalate complexes, log K_1 values. (This figure is reprinted from Williams, R.J.P. *Biol. Revs. Cambridge Phil. Soc.* 28: 381, 1953, with permission.)

ligand groups H_2O, R—OH, and R—CO_2^- for coordination to a cation the hydroxyl group only appears to be able to displace water in the complexes of the very large cations such as lead, barium and calcium and is probably not bound or is much less tightly bound in the complexes of magnesium. The competition between two types of oxygen groups is also shown in the formation of sulfato-complexes, for there is now some evidence that whereas copper and nickel form complexes of the same stability with sulfate ions the type of complex formed in the two cases is very different (11). In the nickel complex the sulfate is bound outside the hydration sheaf (outer sphere complex or ion pair), but in the copper complex it is adjacent to the cation (inner sphere complex). In this respect it seems that the copper ion is behaving like the calcium ion, which also would appear to form inner sphere complexes with sulfate, whereas the nickel ion is behaving like the magnesium ion in remaining hydrated. There is a peculiarity about the cupric ion—it is not spherically symmetrical. It has a radius close to that of nickel in one plane and one like that of calcium in the plain perpendicular to it. This gives it a remarkable flexibility in reaction which is common to very few other cations.

We have discussed the effect of the steric factors such as these in the specificity of metal cations in biological systems pointing particularly to the cases of enolase (7) and of adenosine triphosphatases. We must consider that differences between potassium and sodium ions are also based on differences in size. We return to this point below. Before going on, there is a corollary to the specificity of metal cation action if it is based on structural differences in the formation of the complexes. This point applies no matter whether this difference in structure arises from electronegativity or size differences. The stability of the complexes of different cations need not be related to their efficiency in bringing about chemical reaction. However, when cations form complexes of identical structure then stability and efficiency in catalyzing reactions may well be directly related. In model systems the latter situation is most general as the different cations are present with little or no possible variety in coordinating ligands. In biological systems an immense variety of coordinating possibilities is presented to the cation, and orders of complex stability have never been shown to compare with the catalytic efficiency of cations in these systems (13, 15). Again, there is no known case of a complex of potassium which is more stable than that of sodium, at least for a ligand of plausible significance in a biological system; yet potassium has been shown to be of peculiar importance in biological systems.

Now we have touched upon the two most important factors controlling complex stability. However, there are further factors which we will first list and then discuss.

b) Factors Controlling Stability Sequences

One might well ask what it is that makes stability sequences so irregular. Clearly we must consider:

a) electrostatic attraction forces—the sizes and charges of the central ion and the ligands. Here we include, too, repulsion between like charges and similarly oriented dipoles.

b) Repulsion due to the overlap of filled electron shells. This repulsion is responsible for steric hindrance.

c) Polarization of the anion by the cation, or covalent

bond formation. Here we include electronegativity effects.

d) Polarization of the cation—which is naturally relatively small compared with polarization of the ligand. Here we include ligand field theory.

e) The entropy of complex formation.

Even a brief look at stability constant data shows that the effect of these five terms is sometimes to produce one stability order and sometimes another. For some cations an electrostatic model is adequate, while for others it is completely misleading. As a result different authors when writing on the stability of complexes are inclined to stress the different factors differently. At the end of the article a summarizing reference (16) is given so that a quick appreciation may be had of these viewpoints.

c) *Competition with the Proton*

Selectivity between coordinating groups is not just controlled by the stability constants of the cations in aqueous solution for there is always competition for the coordinating center with a second cation, the proton. The proton behaves a little like a first row transition metal cation but there are many differences. It has a high affinity for ammonia, OH^-, SH^- and a rather lower one for carboxylate groups. The proton differs from other cations in two major respects. It does not readily form chelate complexes. It has one bonding orbital and, as it has no core, it is not polarizable. It cannot act as an electron donor. Thus we observe that the stability order of the proton with nitrogen bases is ammonia > imidazole > aniline > pyridine while the order for a cation like cupric is imidazole $\geqslant$ ammonia > pyridine > aniline. The unsaturated ligands are stabilized relative to the saturated ligands in complexes as we go from the proton to the cupric ion. The change of order is explicable on the grounds that imidazole and pyridine complexes are stabilized by the electron donor properties of the cupric ion, the electrons of the cation going partly into the unsaturated states of the ligand. In a solution in which there is an equal excess of imidazole and ammonia molecules over cupric ions and at a pH above 10.0, there will be equal formation of the ammine and the imidazole complex, but at a pH around 7.0 the cupric ion will be more able to form imidazole complexes because of the much reduced concentration of ammonia molecules at this pH. The effect of the competition between protons and metal ions has been discussed in relation to biological systems (3, 13).

MATCHING OF LIGAND PROPERTIES AND CATION PROPERTIES

So far we have shown that there are some general factors controlling ligand-cation interaction. Associated with the cations which form partly covalent bonds are sets of orbitals which influence the energies of the different possible ligand geometries about a cation. Some ligands are associated with a fixed geometry. It is interesting to see how the two geometries can be matched so as to control selectivity of complex formation. We start from the consideration of some steric hindrance problems.

The simplest effect of steric hindrance is upon coordination number. That the effect is not easily described can be seen by reference to the zinc complexes. Whereas zinc forms a six coordinate hydrate, it forms a four coordinate complex with ammonia, chloride ions and thio groups. When chelate groups coordinate to zinc there is a tendency to revert to six coordination even when the groups are substituted ammines, e.g. ethylenediamine. Generally, chelation involving five membered rings reduces steric hindrance, but it is further reduced if the chelating group is unsaturated, e.g. dipyridyl. In zinc complexes, then, the balance between the coordination numbers is finely adjusted.

The stages of complex formation of the zinc ethylenediamine complexes are compared in Table 2 with those of the zinc ammonia, dipyridyl and phenanthroline complexes. We see that not only size factors control coordination numbers but that the geometry of the ligand is important. The matching of the geometry of the ligand with the preferred polarization of the cation introduces a cooperative effect into selective action. For example, it is rather difficult to pack six ligands of the size of sulfur around a transition metal such as zinc, but four fit easily and match the direction of polarization (sp^3 hybrids) of the zinc orbitals. The nickel orbitals which are less polarizing than the zinc orbitals prefer an octahedral disposition of ligands. The two factors, the lower polarizing power of nickel and its different polarization involving *d* orbitals, lead to a preferential formation of sulfur ligands with zinc rather than nickel. We have discussed the importance of this observation in biological systems (13, 15), and, recently, we have shown its special importance to the zinc containing protein, carboxypeptidase (12, 18) where there is evidence that zinc is bound to sulfur. Suitable observations in other zinc containing proteins are not yet available though zinc-sulfur binding is a distinct possibility. We expect zinc to be four or five coordinate in these complexes. It should be observed that these subtle differences in complexing imply that there is little difference in complexing power between cations of similar ionic radius and electronegativity and that, therefore, cations can be substituted one for another in many *in vitro* enzymes of these metals where the metal acts only as a complexing and not as an electron transfer agent. *In vivo* competition between complexing centers hides the simplicity of these systems, e.g. enolase and carboxypeptidase.

Now, Table 2 also indicates another mixed ligand geometry cation-polarizability effect. We note that the

TABLE 2. *Step Stability Constants of Zinc Complexes, log K Values*

	K_1	K_2	K_3	K_4	K_5	K_6
Ammonia	2.37	2.44	2.50	2.15	0.0	0.0
Ethylenediamine	5.9		5.1		1.9	
Dipyridyl	5.4		4.4		4.1	
o-Phenanthroline	6.4		5.7		4.9	

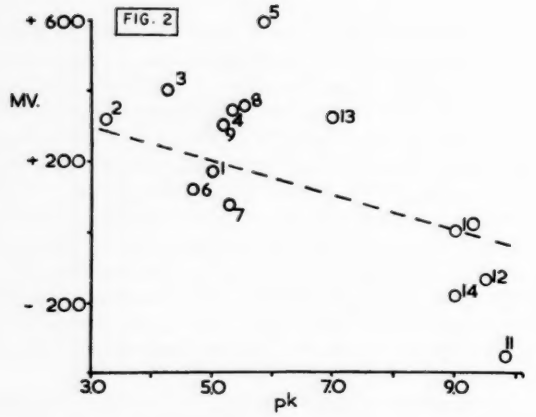


FIG. 2. The redox potentials in millivolts (MV) of some copper complexes: 1. phenanthroline; 2. 5-nitrophenanthroline; 3. 2-chlorophenanthroline; 4. 2-methylphenanthroline; 5. 2,9-dimethylphenanthroline; 6. 2,2′-dipyridyl; 7. 4,4′-dimethyl,2,2′-dipyridyl; 8. quinoline; 9. pyridine; 10. ammonia; 11. ethylenediamine; 12. glycine; 13. imidazole; 14. histidine. The abscissa gives the acid dissociation constant of the basic centers of the ligands.

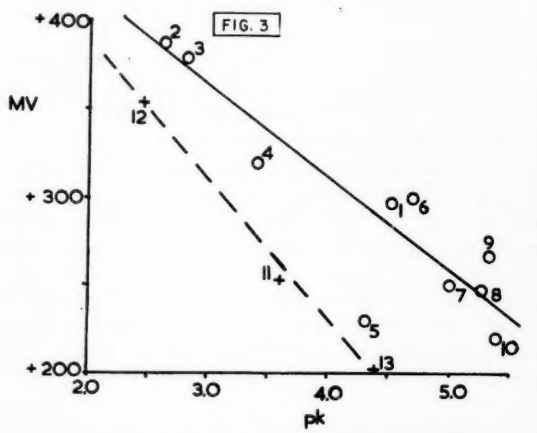


FIG. 3. The redox potentials in millivolts (MV) of some copper complexes, formula type ML_2, where L is a substituted 1-10, phenanthroline: 1. *no* substituent; 2. 4,7-dichloro; 3. 5-nitro; 4. 5-chloro; 5. 4,7-diphenyl; 6. 5-methyl; 7. 5,6-dimethyl; 8. 5-amino; 9. 3,5,6,8-tetramethyl; 10. 4,7-dimethyl. Or a substituted dipyridyl, 11–13; 11. *no* substituent; 12. 4,4′-dicarboethoxy; 13. 4,4′-dimethyl. The abscissa gives the acid dissociation constants of HL.

coordination number induced by the unsaturated ligands is not the same as that of the saturated ligands. This type of consideration has an important effect upon the redox potentials of the copper, Cu(I)/Cu(II) couples. In Figure 2 we illustrate this by comparing ethylenediamine, ammonia, dipyridyl and phenanthroline couples. With simple saturated amines, ammonia and ethylenediamine, cuprous is 2 coordinate. There is no stabilization due to chelation in the ethylenediamine complex as there is no chelation. The cupric complexes are 4 coordinate and in the case of ethylenediamine are chelated. The result is a marked stabilization of cupric relative to cuprous on going from the ammonia couple (*10*) to the ethylenediamine couple (*11*). The same effect is observed on going from imidazole (*13*) to histidine (*14*) couples or from ammonia (*10*) to glycine (*12*) couples. We observe here that the coordination number of the larger cation [Cu(I)] is less than that of the smaller cation [Cu(II)]. A second factor influencing the redox potential is unsaturation of the ligand. The quinoline (*8*) and pyridine (*9*) couples favor cuprous rather than cupric and with quinoline cuprous can form the higher coordinate complex. The difference in potential between the imidazole (*13*) and the ammonia (*10*) couples is also in part due to the unsaturation of imidazole. On going from pyridine (*9*) to dipyridyl (*6*) or phenanthroline (*1*) there is but a small change in redox potential as both cations can now be 4 coordinate and chelated. The change from the ammonia (*10*) to the ethylenediamine (*11*) couple is much larger. From these observations it is clear that when cuprous is bound to unsaturated ligands it will tend to increase its coordination number to four. If the geometry of a protein should provide it with two or three neighbors including imidazole groups of histidine it seems reasonable to expect that the cuprous ion will have a high affinity for such unsaturated ligands as carbon monoxide and oxygen.

There are now five reasons for supposing that cuprous may be bound to histidine groups of some proteins. They are:

1) the chemistry of cuprous is very like that of argentous. Argentous is thought to be bound to histidine in maltase (10).

2) The affinity of both cupric and cuprous at pH 7.0 is for imidazole rather than amino groups.

3) 1:1 Cuprous histidine complexes have a high affinity for carbon monoxide (James and Williams, unpublished).

4) The redox potential of some copper proteins is ~390 mv (5) which is about correct for a copper-imidazole ammine complex of coordination number two (or three).

5) The absorption spectrum of one of the copper proteins, erythrocuprein, in the cupric state has a maximum around 600 mμ (low extinction), but around 350 mμ there is no maximum in the cuprous or cupric complex (5) indicating nitrogen as a ligand atom rather than sulfur. With such a high redox potential it is unlikely that the copper is bound to simple anions. Copper-sulfur bonds (6) are indicated by the yellow color of tyrosinase and by the spectrum of cuprous hemocyanin.

The effect of substitution in the phenanthroline ligand upon the properties of the cuprous and cupric complexes is also instructive. In those cases where there is a 2 or 9 substituent the redox potential is very high and therefore, cuprous is relatively stabilized, ligands (*3*), (*4*) and (*5*), Figure 2, and diquinolyl. The explanation is simple.

The cuprous complexes are tetrahedral whereas the cupric complexes are most stable in a tetragonal configuration. In the tetragonal configuration there is much steric hindrance even before the methyl groups are introduced, whereas in the tetrahedral complexes there is none. The more groups ortho to the nitrogen coordinating centers, the greater the steric hindrance in the cupric complex, the higher the redox potential. The effect of substituents elsewhere in the molecule is largely due to changes in basicity of the nitrogen atoms. This is shown in Figure 3 both for dipyridyls and phenanthrolines. This result is in good accord with the general expectation that good donors of electrons will stabilize the higher valence states preferentially so long as the ligand atoms are small (no steric effects). A third interesting substituent effect is that of 4,7-phenyl groups. In this case there is steric hindrance in the ligand alone (over-crowding) and not in the coordination sphere (Fig. 4). The effect of the steric hindrance between the substituent and the phenanthroline ring system is to swing the plane of the substituent phenyl group away from that of the phenanthroline and hence the phenyl group behaves not as π-acceptor but rather as a σ-donor. It increases the acid dissociation constant and favors cupric rather than cuprous, whereas the effect of such a substituent in the absence of steric effects, e.g. in the 4,4′-diphenyl dipyridyls as compared with dipyridyl itself, is to alter relative stability in favor of cuprous. The important point here is that if an unsaturated group is being brought into combination with any other group which can form π-bonds with it the steric restrictions upon its disposition are of the greatest importance. Consider the case of an unsaturated ligand e.g. a pyridine group, approaching the coordination sphere of a cobaltic or a ferrous ion complex where the central metal ion is held by four groups in a plane. This is the situation in both vitamin B_{12} and in iron porphyrin complexes. The incoming group can lie with the plane of the pyridine in the same plane as a perpendicular to the plane of the porphyrin ring and including the line of the iron coordination N—Fe—N (there are two such possibilities) or, the other extreme, it can lie at 45° to this plane when the steric hindrance between the pyridine hydrogens and the porphyrin ring is minimal.

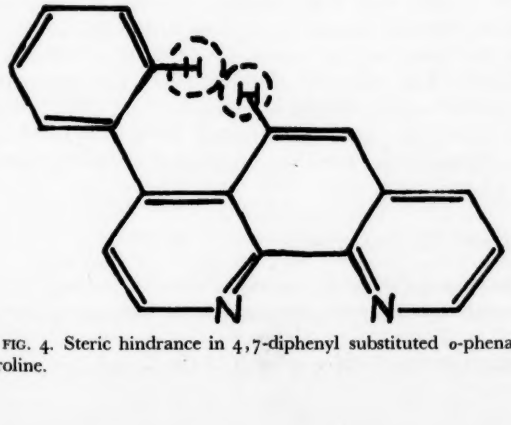


FIG. 4. Steric hindrance in 4,7-diphenyl substituted *o*-phenanthroline.

Structural studies have already shown that in vitamin B_{12} it is partly the steric requirement which controls the orientation of the plane of the incoming ligand, here benzimidazole not pyridine (4). It appears as if the orientation is rather different in ferric myo- and hemoglobin (see below). The exact orientation of the ring of the imidazoles in both cases will influence the oxidation reduction potential of the ferrous-ferric and cobaltous-cobaltic couples for the same reasons as those discussed in the case of the cuprous-cupric complexes. However, in the iron porphyrin proteins and in vitamin B_{12} other steric requirements must also be satisfied—those of the configuration of the protein or saccharide chains. Steric demands due to the groups not immediately linked to the metal cations will affect both the angle of the imidazole ring, α, its tilt, β, and the interatomic distance between nitrogen and iron atoms (see Fig. 7). We have discussed previously the effect of changes in the last factor but will mention it again because of its very great importance in iron complexes. This is again a case of matching the ligand geometry and the cation angular polarization.

HIGH AND LOW SPIN STATES

So far, we have discussed each cation as if it had a single set of parameters which could be used to understand its properties in all circumstances. We have used the parameters size, electronegativity, ionization potential and have referred to electron configuration. Now transition metal cations have a duplicity of nature—they are typical schizophrenics. In certain situations—for example the ferrous ion in aqueous solution—they have the following properties

Fe^2	4 unpaired electrons	rapid exchange of ligands	para-mag-netic	weakly colored	"radius" 0.75

The ion is described as of "high" spin. In other circumstances—when bound to six imine nitrogen atoms, for example—the electrons of the core all pair. The properties of the ferrous ion are now

no unpaired electrons	slow ligand exchange	diamag-netic	usually highly colored	"radius" 0.65

The low spin state has a considerably greater electronegativity than the high spin state.

STERIC HINDRANCE AND SPIN STATE

The spin state of either ferrous or ferric ions in complexes is decided by the character of the ligand. The cyanide ion and *o*-phenanthroline drive both the cations to a low spin condition. In both cases the change from high spin (ionic) complexes to low spin (covalent) complexes induced by basic unsaturated ligands increases the redox potential in favor of the ferrous state.

This is harder to appreciate in the case of the cyanide couple as the simultaneous effect of the negative charges of the ligand depresses the potential (see 14). Now in the low spin state the inter-atomic distance Fe(II)—N is considerably shorter than in the high spin state—probably by as much as 0.10 Å. If through steric hindrance the ligand groups are prevented from coming closer to the cation, then the low spin state cannot be brought about. This is illustrated by the case of 2-methylphenanthroline where the ferrous complex is paramagnetic (high spin). The redox potential of this couple is very low as compared with that of the ferrous/ferric phenanthroline couple itself. There are then two requirements for low spin complexes: *1*) ligands must be "unsaturated", and *2*) basic ligands must be small enough to form 6-coordinate complexes with short interatomic distances.

TABLE 3. *Effect of Steric and Basicity Factors on Spin States*

Low Spin 100% (Approximately)	Sterically Prevented from Low Spin	Basicity Restricted from Low Spin
Cytochrome b series	Hemoglobin series	[Cytochrome a_3 (?)]
Cytochrome c series	Myoglobin series	[Cytochrome a_2]
[Cytochrome a (?)]	Chlorochluorin	Catalase
	Cytochrome d series	
	Cytochrome f series	
	[Peroxidase]	

N.B. Whereas the cytochromes b and c are virtually 100% low spin in both ferric and ferrous states, all the other porphyrin complexes in Table 3 have spectroscopic and/or magnetic properties which demonstrate that they exist as a mixture of high and low spin states (14) (15) (17). The relationship between spin states and physical properties which leads to the above classification has been fully discussed in the references.

SPIN STATE AND LIGAND ELECTRONEGATIVITY

The first way in which the low spin/high spin balance can be shifted in favor of low spin is by an increase of the basicity of the groups which bind the iron in the porphyrin complex. Until recently all the known complexes of ferrous with substituted *o*-phenanthrolines and bipyridyls which did not show either front (or back) steric hindrance gave rise to diamagnetic (low spin) complexes. In the case of the 5,5′-carboethoxy derivative of dipyridyl we have recently obtained a ferrous complex which is paramagnetic but where there can be no question of steric hindrance. This ligand is a very weak base compared with dipyridyl. When we plot the basicity of the ligands against the stability of the ferrous complexes, we find that there is a sharp drop to the 5,5′-carboethoxy compound. When we plot redox potential against acid dissociation constant for these ligands, we obtain Figure 5. There is a maximum in the redox potential plot near the point at which ferrous iron goes from a low to a high spin state. This example is of the greatest consequence. Of the cytochromes and heme-proteins some are high spin and some low spin. We believe that both steric and basicity factors are affecting the spin states and thence redox potentials, and we illustrate this in Table 3. We will now elaborate upon the importance of steric factors.

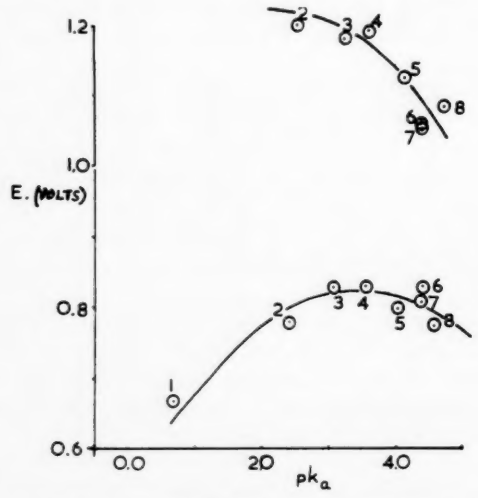


FIG. 5. The redox potentials E. of some ferrous/ferric couples with substituted dipyridyl ligands, L. The top series are for ML_3 complexes of both valence states. The bottom series are for the thermodynamically stable forms of the ferrous and ferric complexes in acid solution, i.e. ML_3 for ferrous and probably $ML_2(OH_2)_2$ for ferric. The abscissa is the acid dissociation constant of the ligand, k_a. The numbering is 1. 4,4′-dicarboethoxy; 2. 5,5′-dicarboethoxy; 3. 4,4′-diphenyl; 4. dipyridyl; 5. 5,5′-dimethyl; 6. 4,4′-diethyl; 7. 4,4′-dimethyl; 8. 4,4′-diethyl-5,5′-dimethyl.

If steric hindrance in the protein prevents the approach of the basic groups to the iron held in the porphyrin, then the effective basicity of the groups at the iron will be reduced and through steric hindrance the iron atoms may remain in a high spin state. The application of this model in biological systems is to the effect of the stereochemical requirements of the protein and the iron in iron porphyrin protein complexes. In the complexes iron already has four unsaturated nitrogen ligands, the porphyrin. If steric hindrance prevents close approach of two further such groups, e.g. imidazoles, as it clearly does in hemoglobin and myoglobin, then we have a high spin iron complex. On the other hand, when two basic groups come closer both iron complexes are low spin and the complexes formed are the cytochromes. The change from low to high spin brings with it several other changes of property (15). Now, there are also other steric requirements of the approach of unsaturated bases to the iron atom due to the formation of π-bonds.

π-BONDS AND D-ELECTRONS

In an octahedral field three of the five *d* orbitals of a transition metal atom are capable of forming π-bonds. This is illustrated in Figure 6. The d_ϵ orbitals match the symmetry of the π orbitals of the ligand. Now such

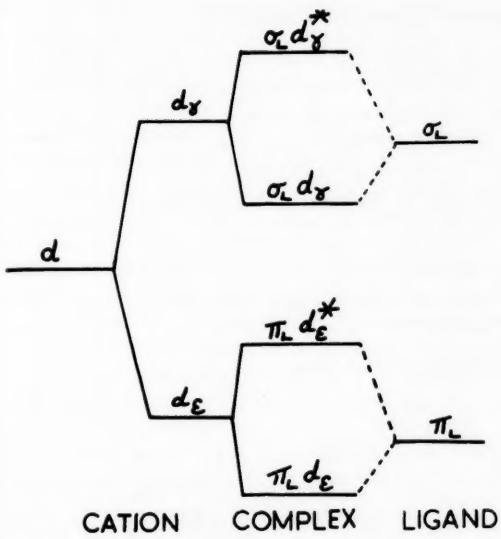


FIG. 6. The molecular orbitals formed from the split d states of a transition metal cation.

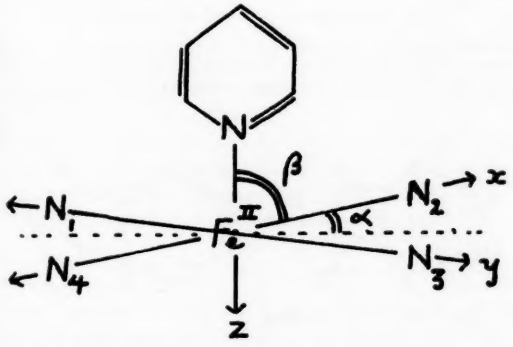
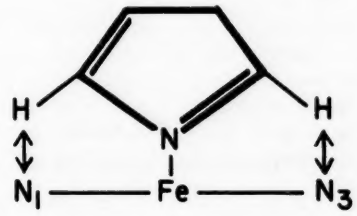


FIG. 7. The orientation of an unsaturated group, here pyridine, with respect to a plane of four coordinating centers.

bonding (which is, for all our purposes, the same as a double bonding in oxygen compounds, such as C⇇O, although we may write it Fe ⇐ N) has important effects upon the stereochemistry of molecules bound to a central cation. If we consider the x,y axis as fixed by four ligands in a plane (see Fig. 7), then the z axis is perpendicular to this plane and the xz and xy orbitals which can form π-bonds are also fixed. In ferrous, ferric and cobaltic complexes these states are either filled or nearly filled with electrons. The minimum repulsion between these electrons and the electrons of pyridine or imidazole and, at the same time, the minimum steric hindrance with the four nitrogen atoms of the porphyrin appears to be when the plane of the pyridine or imidazole is at 45° to the x or y axis. It is of interest to see how observed systems compare with the optimum one. In biological systems we know the orientation of imidazole residues relative to the plane of the porphyrin ring in two cases: (i) *Vitamin* B_{12}. Here the imidazole plane is at an angle of about 40° to the N_1—Fe—N_3 and the deviation from the best possible angle is accounted for in terms of steric hindrance between the imidazole residue and the asymmetric Corin nucleus, i.e. steric hindrance far from the coordination center. (ii) In met [Fe(III)] myoglobin the bond angle between the histidine imidazole and the N_1—Fe—N_3 line is only 10%. This is a very unfavorable configuration. Apart from the overlap of the iron d orbitals with the antibonding π oribtals of the imidazole ring, there is considerable steric hindrance. This can be seen by considering the model:



Unbound N—H Van der Waals distance, ↔, is short. The orientation of the histidine residue in this case is decided, presumably, by the demands of the amino-acid sequence in the peptide chain.

On uptake of oxygen by myoglobin [Fe(II)] further bond shortening must occur between the iron and the histidine. The bond shortening arises from the change to the low spin [Fe(II)] state. Despite this change oxymyoglobin, myoglobin, and metmyoglobin have identical structures. We conclude that the orientation of the imidazole group attached to the protein is not changed greatly on oxygen uptake. As Perutz has pointed out the myoglobin structures are different from those of hemoglobin. In particular methemoglobin and oxyhemoglobin have the same structure whereas hemoglobin has a different structure. Now the ferrous ion in oxyhemoglobin (strong field) is of the same size as the ferric ion in methemoglobin (weak field), both of these ions being smaller than the ferrous ion in hemoglobin (weak field). The change in the structure on oxygenation, which is surely responsible for the cooperative addition of the four oxygen molecules to hemoglobin, is thus related to the change in the imidazole-iron distance or possibly to the concomitant change in the steric arrangement of this imidazole group relative to the porphyrin plane.

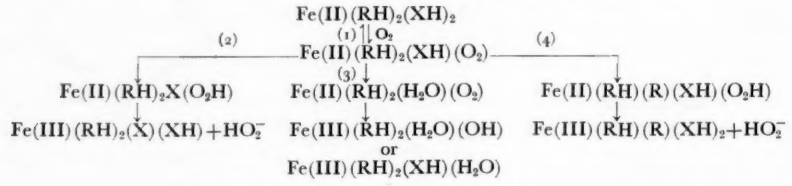
High spin/low spin problems also affect molybdenum chemistry. Recently, Mr. Mitchell and myself have started an extensive survey of the molybdenum compounds with organic ligands. We have found that molybdenum(V) forms a great variety of complexes with different ligand groups such as dipyridyl, 8-hydroxyquinoline, and phenols. Some of these complexes are diamagnetic, through dimerization, and others paramagnetic. The molybdenum(V) valence state has some properties in common with the Fe(III) valence state and

TABLE 4. *Types of Oxidation-Reduction Reactions of Coordination Complexes*

Class of Reaction	Oxidation/Reduction Attack	Reverse Reaction	Example
(i) Labile → Labile	attack by coordinating group	the same	Catalase Compd. I
(ii) Labile → Non-labile	attack by coordinating group	electron transfer	Peroxidase Compd. II Cytochrome a (a_3)
(iii) Non-labile → Labile	electron transfer	attack by coordinating group	B_{12}(?)
(iv) Non-labile → Non-labile	electron transfer	electron transfer	Cytochromes c and b

N.B. In the text (i) is referred to as (a) and (iv) as (b). The characteristics of (ii), which must be followed by (iii) in a reversible catalytic cycle, and (iii), which must likewise be followed by (ii), are a combination of (a) and (b).

TABLE 5. *Scheme of Reactions for Iron/Oxygen Complexes*



	(1) only	(1) then (2)	(1) then (3)	(1) then (4)
Known model example	$Fe(II)(DMG)_2(imid)_2$	$Fe(DMG)_2(N_2H_4)_2$	$Fe(DMG)_2(NH_3)_2$	$Fe(NiOx)_2(imid)_2$
Biological example	Hemoglobin			Cytochrome a

In the table DMG is dimethylglyoxime and NiOx is 1,2-cyclohexanedione dioxime. It is possible to write a very similar scheme for the reactions of iron complexes with H_2O_2 replacing Fe(II) by Fe(III) and Fe(III) by Fe(IV). In such a case the reactions of peroxidase can be written as (1) then (3). RH could well be a porphyrin, the H being at the methene bridge. In some cases water is produced and not peroxides.

it may be that this parallel will be useful in the comparison between molybdenum and iron requiring flavoproteins. Molybdenum(V) compounds can act both as oxidizing and as reducing agents.

REACTION MECHANISMS

Here we do not propose to discuss the properties of Lewis acid catalysts (15). We will refer to oxidation-reduction reactions. There are two main groups of such reactions involving metal ions (Table 4): *a*) direct charge transfer from the cation to the ligand which acts as substrate, e.g. the oxidation of phenolate ions by ferric ions; *b*) mediation of redox reactions in which the metal complex merely transports electrons from one place to another and none of the groups, ligands, of the cation undergo permanent oxidation or reduction. The two cases differ in that case *a*) demands the rapid exchange of ligands in the coordination sphere of the cation while case *b*) requires that there is no such exchange. We deal with case *a*) first.

The reactions of hydrogen peroxide and of oxygen are typical of those reactions which require the immediate association of the metal ion and the reagent in the primary coordination sphere. This is shown by *1*) oxygen will not oxidize certain low spin ferrous complexes without prior dissociation, e.g. cytochrome c and the ferrous dimethylglyoxime dipyridine complex; *2*) hydrogen peroxide is not decomposed by low spin complexes of ferrous and ferric ions, such as peroxidase hydroxide and cytochrome c which do not undergo

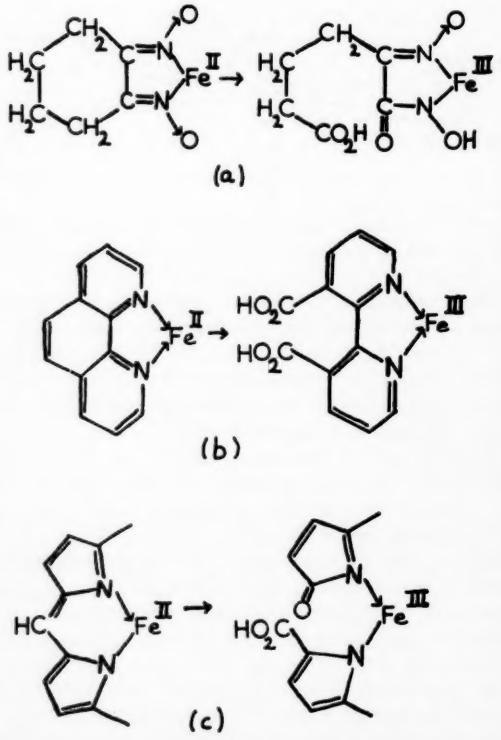


FIG. 8. The oxidation of some ferrous complexes by molecular oxygen.

rapid dissociation reactions; *3*) even when oxygen combines with low spin iron it does not oxidize it unless the oxygen is subsequently readily lost. The further reactions of the initial, intermediate, complexes are also interesting. In the case of the hydrogen peroxide reaction with catalase, Dr. A. S. Brill and myself (2) have suggested recently that the primary step of association Fe(III)—$^{-}O_2H$ is followed by an attack on the porphyrin ring (2). This reaction is very like that of the attack of oxygen on metal porphyrin complexes or, indeed, of the attack of oxygen on many model iron complexes which pick up oxygen (Table 5). We can consider three similar cases of the last reaction: *1*) the oxidation of hemoglobins to choleglobins; *2*) the oxidation of phenanthroline to dipyridyls; *3*) the oxidation of cyclohexadione-dioxime to break the alicyclic ring. We are engaged at present in a detailed study of the last of these reactions. We now have considerable evidence for the reaction path

$$\begin{array}{l} \mathrm{Fe(II)(imidazole)_2(NiOxH)_2} \\ \quad\downarrow\uparrow \\ \mathrm{Fe(II)(imidazole)(O_2)(NiOxH)_2} \\ \quad\downarrow \\ \mathrm{Fe(II)(imidazole)(O_2H)(NiOxH)(NiOx)} \\ \quad\downarrow \\ \text{repeated oxidations removing hydrogen.} \end{array}$$

The oxidation of the ligand is shown in Figure 8*a* and is simply the loss of hydrogen followed by hydrolysis, finally giving the carboxylic acid derivatives. The reaction provides a model for the oxidations of *o*-phenanthroline and of porphyrins similar to that outlined in (14), see Figures 8*b* and 8*c*.

It also shows that in a compound of the type

$$\mathrm{O_2—Fe(II)—N—conjugated\ chain—CH—R}$$

attack upon the —CH— group can be quite rapid. The attack is presumably of the type

$$\mathrm{HO_2—Fe—N—conjugated\ chain—C—R.}$$

The cytochrome oxidase reaction can be written in this way. Then, providing there is a suitable hydrogen donor, the break-up of the porphyrin ring can be prevented and the initial step of dehydrogenation reversed. We are studying models in the hope that we will be able to clarify the steps of such a mechanism. Now, in all these iron complexes there are several hydrogen atoms which could undergo oxidation. We can use Table 5 to illustrate the possibilities. If the atom in the primary coordination sphere of the iron carries hydrogen, then this hydrogen is likely to be more acidic than any hydrogen in the unsaturated conjugated systems of porphyrins, phenanthrolines, or dioximes. The ionization of this hydrogen to the proton provides the ligand with a negative charge which is most easily transferred through the iron to the oxygen molecule. A group such as a coordinated water molecule, a hydrazine, or a peroxide anion is then more likely to attack than the unsaturated ligand (see Table 5). On the other hand, if the group bound in the immediate coordination sphere is the nitrogen of imidazole or pyridine which cannot ionize (the more acidic hydrogen belongs to the porphyrin), then the latter group is the more liable to attack (Table 5). The most acidic hydrogen of the porphyrin system is probably that of the methene bridge, but in the case where there are substituents in the ring, such as aldehyde groups or saturated ring systems, this may not be true, e.g. cytochrome a and a_2. Removal of hydrogen could now take place at the peripheral group. A similar discussion can be used in the comparison between the reactions of peroxidase and catalase. Dr. Brill and myself (2) have been led to suggest that whereas catalase is a di-carboxylate iron porphyrin complex, peroxidase is an amino iron porphyrin complex with the possibility that the sixth group is carboxylate. The difference in reactivity of the two proteins now follows as a difference in ionization, from the $—NH_2$ group of the peroxidase and the porphyrin ring of the catalase. The mechanism for peroxidase would then be

$$\begin{array}{l} -NH_2-\mathrm{Fe(III)}-\mathrm{Y} \underset{H_2O_2}{\longleftrightarrow} -NH_2-\mathrm{Fe(III)}-{}^{-}O_2H + H^+ \\ \qquad\qquad\qquad\

REFERENCES

1. Ahrland, S., J. Chatt and N. R. Davies. *Quart. Rev. Chem. Soc.* 12: 265, 1958.
2. Brill, A. S. and R. J. P. Williams. *Biochem. J.* 78: 253, 1961.
3. Gurd, F. R. N. and P. E. Wilcox. *Advances in Protein Chem.* 11: 311, 1956.
4. Hodgkin, D. W. In: *Progress in the Chemistry of Organic Natural Products*, Vol. XV. edited by L. Zechmeister. Wien: Springer, 1958.
5. Katoh, S. *Nature, London.* 186: 533, 1960.
6. Klotz, I. M. In: *Mechanism of Enzyme Action*, edited by W. D. McElroy and H. B. Glass. Baltimore: Johns Hopkins Press, 1954, p. 257.
7. Malmström, B. G. and A. Rosenberg. *Advances Enzymol.* 21: 131, 1959.
8. Martell, A. E. and M. Calvin. *Chemistry of the Metal Chelate Compounds.* New York: Prentice-Hall, 1953.
9. Mitchell, P. C. H. and R. J. P. Williams. *J. Chem. Soc.* p. 1912, 1960.
10. Myrbäck, K. *Arkiv Kemi.* 11: 47, 1957.
11. Smithson, J. M. and R. J. P. Williams. *J. Chem. Soc.* p 457, 1958.
12. Vallee, B. L. and co-workers. *Biochim et biophys. acta* to be published.
13. Williams, R. J. P. *Biol. Revs. Cambridge Phil. Soc.* 28: 381, 1953.
14. Williams, R. J. P. *Chem. Revs.* 56: 299, 1956.
15. Williams, R. J. P. In: *The Enzymes*, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 391.
16. Williams, R. J. P. *Annual Report Chemical Society London*, 1960.
17. Williams, R. J. P. *Conference on Haemitin Enzymes.* Canberra: Pergamon Press, to be published.
18. Williams, R. J. P. *Nature, London* 188: 322, 1960.

DISCUSSION

Crystal field theory and the lability of complexes of the first transition series

RALPH G. PEARSON
Department of Chemistry, Northwestern University, Evanston, Illinois

Crystal field theory is applicable to any orderly arrangement of interacting particles to which it is reasonable to assign electrical charges or dipoles. A complex ion would be one example of such a system. The crystal field is the electric field acting at the central metal ion due to the attached groups or ligands. This field has certain symmetry properties which greatly affect the energies of electrons particularly in the various atomic *d* orbitals of the central ion. These energy changes lead to consequences which have been of value in explaining the spectral, magnetic and thermodynamic properties of complex ions of the transition metals. A number of review articles discuss these properties in terms of the theory (1, 3, 5, 7).

Another important consequence of the splitting apart in energy of the several *d* orbitals is that the rates of reaction of similar compounds of metal ions with different numbers of *d* electrons are influenced strongly. The reason for this can be seen from the following considerations: the *d* electrons will occupy preferentially the lower energy *d* orbitals. This makes the complex more stable than otherwise by an amount called the crystal field stabilization energy (CFSE). This energy difference can be estimated from spectroscopic data. It also shows up in the thermodynamic properties such as heats of hydration, lattice energies and stability constants. In the case of a substitution reaction, for example, any reaction mechanism will change the coordination number and geometry of the original reactant as it passes into the transition state. Systems which were strongly crystal field stabilized will usually resist these changes since it was the original coordination number and geometry which produced the CFSE.

This is particularly so for octahedral complexes containing three or eight *d* electrons and six *d* electrons in the special case of spin-pairing of these electrons. This shows up most dramatically in the special inertness towards substitution of complexes of chromium(III), cobalt(III), rhodium(III) and iridium(III). In these cases very regular octahedral complexes tend to form which are difficult to distort for any reaction mechanism. The inertness is also helped by the high positive charge on the central ion.

For the divalent metal ions of the first transition series definite predictions can be made by the use of crystal field theory as to relative rates of reaction. The predictions are that rates of substitution will vary in the order Mn > Fe > Co > Ni < Cu < Zn. In this listing ferrous ion is considered as spin-free (paramagnetic). If ferrous ion is spin-paired (diamagnetic), then, it is predicted to react the slowest of all.

The above predictions can be tested by looking at

experimental data on rates of reaction or, even better, activation energies. The example of the *o*-phenanthroline and dipyridyl complexes is particularly useful since the latter data are available for several of the ions. Table 1 shows some experimental rate data and some theoretical calculations of loss of CFSE for a number of such systems. The reaction studied is the rate of dissociation of one of the ligands from the indicated complex.

$$Fe(phen)_3^{++} \rightarrow Fe(phen)_2^{++} + phen \qquad (1)$$

These rates generally are obtained from the study of isotope exchange reactions. Such exchange reactions of, for instance, labeled phenanthroline with unlabeled phenanthroline have rates independent of the concentration of free ligand. Accordingly, they are considered to be S_N1 (dissociation) reactions and the losses in CFSE are computed for a transition state in which only five groups instead of six are attached to each metal ion (the organic amines are bidentate ligands). The crystal field parameter D_q is found from spectral studies and is about 3–4 kcal per mole for these particular ligands attached to the divalent ions of the first transition series. It can be seen that low rates and high activation energies are found for the metal ions that have a large loss in CFSE.

Most substitution reactions of these metal ions are very much faster and special methods must be used to follow them. A very interesting example is supplied by solvent exchange studies done by means of nuclear magnetic resonance methods. It has been known for some time that paramagnetic ions will shorten the measured transverse (T_2) and longitudinal (T_1) relaxation times in NMR spectra. Recently it has been shown that the changes enable rate constants for exchange reactions of the following kind to be measured (6):

$$[Cu(H_2{}^*O)_6)^{++} + H_2O \rightleftharpoons [Cu(H_2O)_6]^{++} + H_2{}^*O \qquad (2)$$

The asterisk is simply used to indicate a solvent molecule bound initially to a paramagnetic ion to distinguish it from bulk solvent molecules. In general one can measure

TABLE 1. *Kinetic Data and Crystal Field Stabilization Energy Changes for the Dissociation of Bivalent Metal Complexes of Phenanthroline and Dipyridyl at 25° C*

Electronic System	$\Delta E(D_q)$	Complex Ion	$K_{exch.}$ (min^{-1})	$E_{act.}$ (kcal $mole^{-1}$)	$\Delta S^{\ddagger}$ (e.u.)
d^3	2	$V(phen)_3^{++}$			
d^5	0	$Mn(dipy)_3^{++}$	fast		
d^6	4	$Fe(phen)_3^{++}$	0.0043	32.1	+28
		$Fe(dipy)_3^{++}$	0.0089	28.4	+17
d^7	0	$Co(phen)_3^{++}$	12:66	20.6	+5
		$Co(dipy)_3^{++}$	fast		
d^8	2	$Ni(phen)_3^{++}$	0.0005	25.2	+1
		$Ni(phen)^{++}$	0.0011	23.1	−5
		$Ni(phen)^{++}$	0.0005	26.2	+5
		$Ni(dipy)_3^{++}$	0.137	22.4	+2
d^9	0	$Cu(phen)_3^{++}$	fast		
d^{10}	0	$Zn(phen)_3^{++}$	fast		

Data from Ellis, P. and R. G. Wilkins. *J. Chem. Soc.* p. 299, 1959.

TABLE 2. *Lower Limits to Exchange Rate Constants, $1/\tau_B$, for Paramagnetic Ions in Water and Methanol at 25°C*

Ion	$CH_3\underline{O}H$	$\underline{C}H_3OH$	$H_2\underline{O}$*	$H_2\underline{O}$†
Cr^{+3}	$2.1\cdot10^4$	$7.0\cdot10^3$	$1\cdot10^5$	slow
Fe^{+3}	$3.2\cdot10^4$	$2.2\cdot10^4$	$2\cdot10^5$	$1\cdot10^4$‡
Mn^{++}	$2.5\cdot10^5$	$1.8\cdot10^4$	$5\cdot10^5$	$7\cdot10^7$
Co^{++}	$1.1\cdot10^4$	$1.7\cdot10^3$	$5\cdot10^3$	$2\cdot10^5$
Ni^{++}	$2.6\cdot10^3$	$2.2\cdot10^2$	$6\cdot10^3$	$4\cdot10^4$
Cu^{++}	$1.0\cdot10^4$	$1.0\cdot10^4$	$8\cdot10^3$	$6\cdot10^5$
Gd^{+3}	$1.2\cdot10^5$	$2.1\cdot10^4$	$1\cdot10^4$	
Ce^{+3}	$2.5\cdot10^2$	$0.8\cdot10^2$	$8\cdot10^2$	

All rate constants in sec^{-1}. The underlined atoms are the nuclei whose exchange rate (or rate of relaxation) is being measured.

* Rounded off values from Morgan, L. O. and A. W. Nolle. *J. Chem. Phys.* 31 : 365, 1959; except Fe^{+3}, and Ce^{+3}, Conger, R. L. and P. W. Selwood. *J. Chem. Phys.* 20: 383, 1952.

† Connick, R. E. and R. E. Poulson. *J. Chem. Phys.* 30: 759, 1959.

‡ H. Taube, private communication.

either the life-time, τ_B, of a water molecule bound to the ion or the life-time, T_{2B}, of a nuclear spin state in a water molecule bound to the ion (11). Whichever time is the longer will correspond to the rate determining step and will be measured. The reciprocal of τ_B is the first order rate constant for the forward step of reaction 2.

Table 2 presents some exchange data obtained in water and in methanol solutions of some paramagnetic ions. In most cases only lower limits can be put on the rate constants because of the possibility that T_{2B} is rate controlling. Even so, the data are tremendously valuable in that for the first time it is possible to get some idea of the permanence of a solvent molecule bound to the simpler ions. For some cases, such as copper(II) and nickel(II) in methanol, it is clear that the rate of solvent exchange is being measured since the rates are the same for the methyl protons and for the hydroxyl protons. This would not be true if T_{2B} were being measured.

It is of considerable interest that methyl alcohol molecules are exchanged more slowly than water molecules. This is either because they are held more firmly or because the methyl group offers steric hindrance to an incoming solvent molecule in an S_N2-like process. The very high lability of solvent attached to iron(III) is noteworthy. The results are probably complicated by the fact that, even in the acid solutions where the spectra were taken, some $[Fe(H_2O)_5OH]^{++}$ will exist, and it is known that the hydroxy group is usually strongly labilizing for substitution reactions (10). Even so, the water exchange rate constant is clearly very much greater than for an ion such as $[Cr(H_2O)_6]^{+3}$ where τ_B is about 10^6 seconds instead of 10^{-1} This is an extreme example of crystal field stabilization in the latter ion.

Table 3 presents some data on the sulfation reaction obtained by Eigen (4) using ultrasonic relaxation spectroscopy. It is strongly indicated by the results that the process of forming a sulfato complex from an aquo complex consists of several stages. The first of these are

TABLE 3. *Rate Constants for Reaction* $[M(H_2O)_n]^{++}, A^{--} \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ $[M(H_2O)_{n-1}A] + H_2O$ *at 25°C*

M	A	k_1, sec^{-1}	k_2, sec^{-1}
Be^{++}	SO_4^{--}	1×10^2	1.3×10^3
Mg^{++}	SO_4^{--}	1×10^5	8×10^5
Ca^{++}	SO^{--}	(10^7)*	(10^8)*
Mg^{++}	$S_2O_3^{--}$	1×10^5	1.5×10^6
Mg^{++}	CrO_4^{--}	1×10^5	1.5×10^6
Zn^{++}	SO_4^{--}	1×10^5†	1×10^7†
Cu^{++}	SO_4^{--}	(10^4)*	1×10^6
Ni^{++}	SO_4^{--}	1×10^4	1×10^5
Co^{++}	SO_4^{--}	2×10^5	2.5×10^6
Mn^{++}	SO_4^{--}	4×10^6	2×10^7

Data from reference (4).
* The numbers in parentheses are relatively uncertain.
†M. Eigen, private communication.

concerned with the diffusion controlled formation of an ion-pair or outer sphere complex.

$$[M(H_2O)_6]^{++} + SO_4^{--} \rightleftharpoons [M(H_2O)_6]^{++}, SO_4^{--} \qquad (3)$$

The last stage, and the one of chemical interest, is the rearrangement of this ion-pair to the true complex.

$$[M(H_2O)_6]^{++}, SO_4^{--} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [M(H_2O)_5SO_4] + H_2O \qquad (4)$$

The table shows values of k_1 and k_2 for a number of metal ions. Some data for anions other than sulfate are also given.

The results show the expected dependence on electrostatic factors since small cations react more slowly than similar but larger ions. The transition metal ions show the expected order manganese(II) > cobalt(II) > nickel(II) < copper(II) < zinc(II). Two other features are especially noteworthy: one is that the rate constant k_1 is apparently independent of the anion associated with the cation, and the other is that the value of k_1 is remarkably similar to the rate constants for water exchange given in Table 2. This seems to be very powerful evidence for an S_N1 or dissociation mechanism operating in all these cases so that the rate step is the loss of a water molecule from the first coordination shell of the cation. Apparently, the anion gives very little assistance in pushing off the leaving group.

As a final example, we may take the rates of dissociation, or of exchange, of ethylenediamine with some metal ions.

$$Ni(en)_3^{++} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ni(en)_2^{++} + en \qquad (5)$$

The rate constant k_1 for nickel(II) has been measured by Wilkins and Ahmed (12) using a stopped flow method. Its value is 89 sec^{-1}. The ratio k_2/k_1 is equal to K_3, the third formation constant of nickel(II)—en which is equal to 1×10^5. This makes k_2 equal to 8.9×10^6 M^{-1} sec^{-1}. These values of k_1 and k_2 are valid only in excess acid where enH^+ is the reagent.

It is of interest to compare the en rates with nickel ion with those obtained for copper(II) and cobalt (II) by an NMR technique involving direct measurement of T_1 and T_2 of water protons in solutions containing excess en and the metal ions (8, 9). The mechanism for changing the relaxation times is that of exchange of coordinated en with free en in solution. The protons of the newly released en molecule then exchange rapidly with the protons of water. Probably only one end of the en molecule need be released from the metal ion for proton exchange to occur.

In the case of cobalt(II) the NMR studies (L. O. Morgan, private communication) give a rate of dissociation of 1.3×10^3 sec^{-1} at 25° C for $[Co(en)_3]^{++}$. This is the rate of exchange also and is independent of the en concentration. The number may be compared to that of 89 sec^{-1} for $[Ni(en)_3]^{++}$. The case of copper (II) is quite different in that the exchange reaction is a bimolecular process, the rate being given by

$$\text{Rate} = 2.4 \times 10^7\ M^{-1}\ sec^{-1}[en][Cu(en)_2^{++}] \qquad (6)$$

At reasonable ethylenediamine concentrations, this is much more rapid than either the nickel or cobalt rates. The activation energy for process (6) is 4.5 kcal, which is very low.

The different behavior of copper ion is associated clearly with the lower coordination number and tetragonal structure of copper complexes. The water molecules above and below the plane of $Cu(en)_2^{++}$ can be displaced easily by en molecules acting as unidentate ligands. A rearrangement process then occurs which makes the added en molecule identical with one or both of the en molecules in the plane. Reversal of the process leads to a random loss of en equivalent to exchange. There is a close similarity of this process to the postulated reaction mechanisms of square planar complexes in general (2). Such an S_N2 path probably always accounts for the high lability of copper(II) complexes. In the case of the highly hindered 2,3-diamino-2,3-dimethylbutane (tetramethylethylenediamine or tetraMeen) both nickel and copper add only two molecules of ligands forming square complexes. The nickel complex, for example, is diamagnetic as expected in a square complex. Here, where similar mechanisms may be expected for both metal ions, measurements of the dissociation rates show that the copper complex reacts 2000 times faster than the nickel complex at 0° C (13). The activation energy for nickel (II) is four kilocalories higher than for copper(II). This is in the direction predicted by crystal field theory.

REFERENCES

1. Basolo, F. and R. G. Pearson. *Mechanisms of Inorganic Reactions.* New York: Wiley, 1958, chap. 2.

2. Basolo, F. and R. G. Pearson. *Mechanisms of Inorganic Reactions.* New York: Wiley, 1958, chap. 6.

3. Dunn, T. M. In: *Modern Coordination Chemistry*, edited by R. G. Wilkins and J. Lewis. New York: Interscience, 1960, chap. 4.
4. Eigen, M. *Z. Elektrochem.* 64: 115, 1960.
5. George, P. and D. S. McClure. In: *Progress in Inorganic Chemistry*, edited by F. A. Cotton. New York: Interscience, 1959, chap. 6.
6. McConnell, H. M. and S. B. Berger. *J. Chem. Phys.* 27: 230, 1957.
7. Moffitt, W. E. and C. J. Ballhausen. *Ann. Rev. Phys. Chem.* 7: 107, 1956.
8. Morgan, L. O. and P. F. Cox. *J. Am. Chem. Soc.* 81: 6409, 1959.
9. Morgan, L. O., J. Murphy and P. F. Cox. *J. Am. Chem. Soc.* 81: 5043, 1959.
10. Pearson, R. G. and F. Basolo. *J. Am. Chem. Soc.* 78: 4878, 1956.
11. Pearson, R. G., J. W. Palmer, M. M. Anderson and A. L. Allred. *Z. Elektrochem.* 64: 110, 1960.
12. Shamsuddin Ahmed, A. K. and R. G. Wilkins. *J. Chem. Soc.* p. 3700, 1959.
13. Wilkins, R. G. *J. Chem. Soc.* p. 4521, 1957.

GENERAL DISCUSSION

Dr. Frank A. Cotton (Massachusetts Institute of Technology): Dr. Williams, it seems in principle that one could imagine, in cases of the type you mentioned, that when a change in conformation of a remote part of the molecule necessitated by chelation occurs, reactivity in a part of the molecule remote from the chelating atom would be affected. I wondered if you have any model systems in which just this happens.

Dr. Williams: In the case of vitamin B_{12}, which has a strong absorption band at 350 mμ, there is a cobalt atom bound by four nitrogens in a planar ring system, with groups x and y above and below the ring. If x and y are cyanide, then this band is of very high intensity. If you now change the cyanide and substitute benzimidazole, which is the usual B_{12} derivative, for cyanide the 350 band drops somewhat in intensity, which is surprising because the cyanide is usually the lowest intensity, and a new band starts to appear at about 310 mμ. If you now put in a second bulky benzimidazole group and remove the second cyanide, this band disappears. Now we theorize, I think you will allow this argument; this 350 band can be associated with the conjugated system. Because of the steric hindrance of the benzimidazole, the conjugated ring is distorted and twisted out of shape, so that the length of the conjugated chain is now very much shorter. This effect would give you a band about 300 mμ.

Dr. Cotton: Well, that is even better than a model system. That is a real one.

Dr. Williams: I would like to get some additional evidence of this type from another point of view.

Dr. Albert: Dr. Williams speaks of the physiological or biological pH of 7, and, of course, one must take some particular figure for general remarks like this. It is true, also, the pH of the blood is known to be exactly 7.3, but we know that other physiological fluids vary somewhat from this. Tears are said to be 8.5 and pharmaceutical manufacturers take great care to make eye lotions slightly alkaline. They are then more soothing. Urine is about pH 5. In recent years it has been shown that inside most mammalian cells the pH is 6.8 (Aldridge, W. N. *Biochem. J.* 67: 424, 1957). Now this is significant because it means we have different pH in different parts of the body and this does give us the opportunity for getting specificity in pharmacological action. I think it has been known even longer that the pH of plant cell crushes is also quite acidic, in the pH 5 region (Thomas, M. *Endeavour* 10: 160, 1951).

Dr. Williams: I would like to make the point that, in this discussion of the biological systems, I treat the system as if it were in thermodynamic equilibrium; i.e. everything is assumed to come to equilibrium. This is a basic starting point in the comparison of stability constants with a living system. In general, in the body you are not in thermodynamic equilibrium. It is a system which is kicking over, a changing system, and there will be different regions of the system at specific pH values. All the same, as most chelation reactions are very, very fast, this won't matter very much in the separate regions, even though the different regions may differ in all respects. You will get a good approximation to the thermodynamic situation in each region. On the other hand, it is quite clear that the distribution of elements like sodium and potassium between the regions is extremely sensitive to the pH difference between regions. It is easiest to show this in model systems where you can compare readily a nonequilibrium system and an equilibrium system.

Dr. Thomas L. Coombs (Peter Bent Brigham Hospital): I would like to ask Dr. Williams if this series or order of stability constants can be extended to other groups of metals.

Dr. Williams: There can be, I think, few other simple generalizations of the kind that I have tried to make. You can generalize to a certain extent with a group of closely related ligands. If, however, you take sulfur ligands and compare them

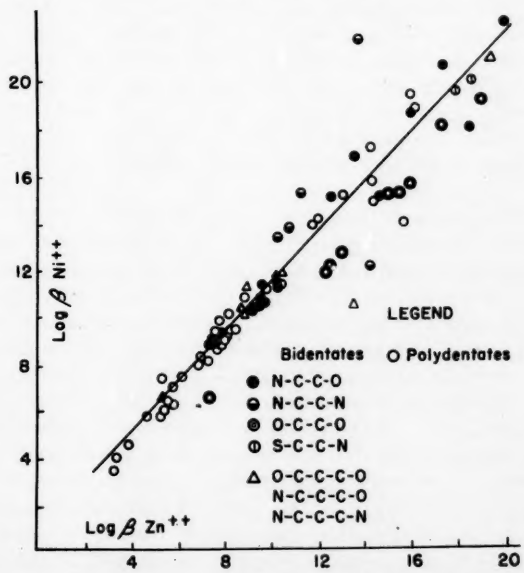


FIG. 1. Correlation of nickel and zinc chelate stability constants.

with nitrogen ligands and with oxygen ligands, and you study 20 metals in the periodic system, you get several different series, not one. You also begin to lose sight of the factors that determine relative stability constants. I think the reason is very simple: in any series other than the Irving-Williams series there is no simple sequence of atomic parameters running along with the stability series. If you take the series of manganese through copper to zinc, then you could give different reasons why you would expect this series to hold—the changing radii of the various cations, the change of ionization potentials, or the crystal field effects plus the Jahn-Teller distortion or entropy arguments. All the factors that one imagines are contributing to the stability constants are going in the same direction. Therefore, they are all contributing to giving you one series. If, however, you take this series and try to put other cations into it, then you find that for one reason or another—perhaps it is the steric hindrance reason, or the polarizability, or its entropy, or perhaps its change from the valence state of the cation to another—there is no single series for all the different physical factors and no simple constant stability sequence.

Dr. Henry Freiser (University of Arizona): Dr. Pearson, I would welcome your comments on the applicability of crystal field theory to a correlation of the nickel and zinc chelate stability constants which we found (Freiser, H., Q. Fernando and G. E. Cheney. *J. Phys. Chem.* 63: 250, 1959) to apply to a wide variety of ligands. By plotting the log β_2 (stability constant) of nickel chelates against the corresponding log β_2 for zinc (see Fig. 1) the following relation was found to apply to most of the ligands (which included 6-membered ring forming species as well as 5-membered ring N—O containing species).

$$\log \beta_{Ni} = \log \beta_{Zn} + 1.7.$$

Similar lines of unit slope were obtained for 5-membered ring N—N ligands (intercept + 3.5), O—O ligands (intercept −2.0) and N—S (intercept −1.5).

Since in nickel complexes there should be a considerable contribution of crystal field stabilization to the stability, one would expect a slope considerably greater than unity. From our correlation it can be shown that the crystal field stabilization energy for nickel would have to be between 2 and 3.5 kcal/mole, which seems rather small.

It might be interesting to note that when sulfur atoms are involved, the zinc chelate is more stable than that of nickel [see also Fernando and Freiser (mercaptopropionic acid) (Fernando, Q. and H. Freiser. *J. Am. Chem. Soc.* 80: 4928, 1958) and (6-mercaptopurine) (Cheney, G. E., H. Freiser and Q. Fernando. *J. Am. Chem. Soc.* 81: 2611, 1959)].

Dr. Pearson: When you told me about this at Urbana one year ago, I couldn't explain it then and I can't explain it now. But perhaps a few comments would be in order. The idea is that nitrogen bearing ligands empirically, say from spectral data, have a higher crystal field effect. Whatever the causes may be do not concern us, but nitrogen bearing ligands have a higher crystal field effect than oxygen bearing ligands. Therefore, one should have relatively more stabilization for ligands containing two nitrogens than for ligands containing two oxygens. That is, nickel should be stabilized with respect to zinc because of the two nitrogens, whereas what Dr. Freiser points out is that if one looks at a large body of data it doesn't seem to be so. The interpretation of your unit slope would be that zinc and nickel seem to vary by a constant amount. Now there are several comments that can be made. One is that nitrogen and oxygen really don't differ very much in their crystal field effect. You will find them sitting side by side in the series. Another is that somewhat similar data can be taken by other people (see McClure, D. S. and P. George. In: *Progress in Inorganic Chemistry*, Vol. 1, edited by F. A. Cotton, New York: Interscience, 1959, p. 381.); they make the case look pretty good for crystal field theory by indicating that the nitrogen bearing ligands do seem to show a greater effect than the oxygen bearing ligands. Except for those comments, I have no explanation for why this unit slope is found.

Estimation of the numerical value and validity of association constants[1]

J. W. GRYDER
Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland

THE ACCURATE DETERMINATION of the constitution and concentration of complex species existing in ionic equilibrium in aqueous solutions is among the most difficult tasks of chemistry. It is the purpose of this paper to review briefly the general method which has been employed in the study of complex species, to emphasize its limitations, and to discuss procedures for checking the reality of the species found to be present and the numerical values of the constants.

Most of the systems containing complex ions which have been investigated were studied by procedures which might be called colligative (2, 4, 7, 16, 19, 21). That is, a single property, for example, the concentration of one of the species existing in the solution, is measured; conservation equations for the total concentration of reacting species are written in terms of the concentration of unbound reacting species and of complex species which might be present, the activity coefficients of the species, and the equilibrium constants for the complexes; finally the equilibrium constants are determined by fitting the equation to experimental data with the aid of some procedure for evaluating activity coefficients. The requirement is made that the constants must be positive and the correct set of constants is to be that set which is smallest in number and which agrees with the experimental data to within the probable error. Two major difficulties are immediately evident. One is that no fully satisfactory procedure is available for estimating activity coefficients (6). The other is that a fit of experimental data does not guarantee that the species found to be present are real entities.

The first of these difficulties is partially eliminated if an extrapolation to infinite dilution is made. However, such an extrapolation tends to decrease the reliability of constants for polynuclear species and, furthermore, the results are dependent upon the particular extrapolation procedure employed. If thermodynamic constants in terms of activities based upon infinite dilution as the standard state are required, the above procedure must be used in spite of its limitations. For many purposes, however, these thermodynamic constants are not required. It is then possible to use a second method for avoiding the difficulties inherent in the variation of activity coefficients (16, 19). This method involves the use of a large excess of inert electrolyte and either the assumption that activity coefficients are independent of variations in the concentration of reacting species or the extrapolation of the results to infinite dilution in reacting species while still keeping some large concentration of inert electrolyte present.

Constants determined by this method frequently are called concentration constants or formal constants. The implicit disparagement of the method contained in the names of the derived constants is unfair. In essence, this approach defines a new standard state based upon infinite dilution of reacting species in constant concentration of supporting electrolyte. Since the choice of standard state is completely arbitrary, the method is as thermodynamically valid as the method based upon the usual definition of standard states (7, p. 189). Since this procedure is much less dependent upon extrapolation procedures, its use should be encouraged. It must be remembered, however, that just as the usual choice of standard states results in constants relative to hydrated species, the new choice will result in constants relative to hydrated species complexed with supporting electrolyte. Since even perchlorate ion has been shown to form complexes with some cations, care is indicated, particularly when a buffer system is used as supporting electrolyte.

Much more important than the difficulty imposed by the variation in activity coefficients is the possibility that species deduced from curve fitting may have no physical reality. With a sufficient number of parameters almost any data can be fitted. Consequently, conductivity and spectroscopic procedures which involve parameters for ion mobilities or extinction coefficients in addition to equilibrium constants are particularly susceptible to false fits. Potentiometry is probably the most precise method for obtaining the requisite data and, in addition, introduces no additional parameters. However, it suffers from the fact that for many systems reversible half-cells cannot be devised. An extension of Bjerrum's concept of corresponding solutions (3, 20) increases the applicability

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of potentiometry for cases in which only mononuclear species are formed (1, 9). For a system containing metal ions M and M′ which form complexes with a ligand L, one can write

$$(L)_0 = (L) + \bar{n}(M)_0 + \bar{n}'(M')_0 \qquad (1)$$

where $\bar{n}$ and $\bar{n}'$ are the average number of ligands bound per metal ion of type M and M′ respectively; $(L)_0$, $(M)_0$, and $(M')_0$ are total concentration of ligand and of metal ion M and M′; and (L) is the concentration of free ligand. If sufficient concentration of inert electrolyte is present to guarantee constancy of activity coefficients and if mononuclear species only are formed, $\bar{n}$ and $\bar{n}'$ are functions of (L) only. Under these circumstances, $\bar{n}$ equals the partial derivative of $(L)_0$ with respect to $(M)_0$ at constant (L) and $(M')_0$ or, since $\bar{n}$ depends only on (L), it is equal to the ratio of the change in $(L)_0$ divided by the change in $(M)_0$ at constant (L) and $(M')_0$. A similar result is obtained for $\bar{n}'$. Symbolically then

$$\bar{n} = \left(\frac{\partial(L)_0}{\partial(M)_0}\right)_{(L),(M')_0} = \left(\frac{\Delta(L)_0}{\Delta(M)_0}\right)_{(L),(M')_0} \qquad (2a)$$

$$\bar{n}' = \left(\frac{\partial(L)_0}{\partial(M')_0}\right)_{(L),(M)_0} = \left(\frac{\Delta(L)_0}{\Delta(M')_0}\right)_{(L),(M)_0} \qquad (2b)$$

By suitable design of experiment it is possible to keep the quantity (L) constant both when one of the metal-ions and the corresponding metal form a reversible half-cell (1) and when the two metal-ions form an oxidation-reduction couple (9). Experimental data can then be used in Equation (2) to calculate $\bar{n}$ and $\bar{n}'$ and these quantities substituted in Equation (1) to yield (L). When $\bar{n}$ is known as a function of (L), standard procedures can be used to calculate equilibrium constants.

In addition to extending the potentiometric method to new systems, the procedure outlined has the advantage that the experimental data can be checked immediately to determine if the assumptions upon which it is based are valid for a particular system. This is done by noting if values of $\bar{n}$, $\bar{n}'$ and (L) obtained by the procedure are indeed constant at widely different values of $(M)_0$, $(M')_0$, and $(L)_0$ but at emf values which should give constant (L) and hence constant $\bar{n}$ and $\bar{n}'$. If constancy is observed, one may conclude that activity coefficients do not vary and that polynuclear species are not formed. After such a test, one feels slightly more confident that the species indicated by the equilibrium constants obtained from curve fitting are real entities. Additional credence is given to the conclusions if the same species are found for different concentrations of supporting electrolyte and for different electrolytes.

Different numerical results for the association constants frequently are obtained when different methods are used for analyzing the experimental data (8, 17, 21). In some cases, particularly when smoothed values instead of experimental data are employed, the species found to be present are dependent upon the method of analysis (17). Undoubtedly, a least squares treatment is most reliable, but even this treatment requires care. For example a least squares analysis of a straight line in the form $y = ax + b$ will give different values for a and b from those obtained by an analysis of the same data in the form $y/x = a + b/x$. This is because the two analyses weight points differently. For experimental data the weighting of points must be determined by the experimental error in the data. This point is emphasized by Rydberg and co-workers (17, 18, 21, 22).

In spite of the care with which colligative procedures are carried out, independent physical means are required to prove conclusively the existence of the species found. In a few cases infrared and Raman spectra have been used to show the presence of complexes and to determine constants (5, 11, 15, 24). In favorable cases X-ray diffraction has been used to determine the structure of ions in solution (23). Nuclear magnetic resonance has been used to measure equilibrium constants in an essentially colligative fashion (15). It is interesting to note that molecular HNO_3 is distinguished from NO_2^+ by a difference in the frequencies of vibration as determined by infrared and Raman spectroscopy (10, 13) whereas in the nuclear magnetic resonance spectrum only a chemical shift occurs when a mixture of NO_2^+ and HNO_3 are present (14). This is because the rate of interchange is slow in comparison with the time of vibration but fast in comparison with the time of nuclear relaxation. For systems in which chemical equilibrium is slowly attained, nuclear magnetic resonance may prove useful for direct verification of species.

REFERENCES

1. Ahrland, S., J. Chatt, N. R. Davies and A. A. Williams. *J. Chem. Soc.* p. 264, 1958.
2. Bjerrum, J. *Metal Amine Formation in Aqueous Solution.* Copenhagen: P. Haase and Son, 1941.
3. Bjerrum, J. *Chem. Abst.* 40: 4590, 1946.
4. Clark, W. M. *Oxidation Reduction Potentials of Organic Systems.* Baltimore: Williams and Wilkins, 1960.
5. Cotton, F. A. In: *Modern Coordination Chemistry,* edited by J. Lewis and R. G. Wilkins. New York: Interscience, 1960, p. 301.
6. Discussions Faraday Soc. No. 24. *Interaction in Ionic Solutions.* Aberdeen: Aberdeen Univ. Press, 1958.
7. Edsall, J. T. and J. Wyman. *Biophysical Chemistry,* Vol 1, New York: Acad. Press, 1958, p. 477, 591.
8. Gryder, J. W. *J. Am. Chem. Soc.* 77: 6196, 1955.
9. Gryder, J. W. *Proc. Nat. Acad. Sc. U. S.* 46: 952, 1960.
10. Ingold, C. K. and D. J. Millen. *J. Chem. Soc.* p. 2612, 1950.
11. Jones L. H. and R. A. Penneman. *J. Chem. Phys.* 22: 965, 1954.
12. Leden, I. *Hoppe-Seyler's Ztschr. physol. Chem.* A188: 160, 1941.
13. Marcus, R. A. and J. M. Fresco. *J. Chem. Phys.* 27: 564, 1957.
14. Ogg, R. A. and J. D. Ray. *J. Chem. Phys.* 25: 1285, 1956.
15. Redlich, O. and G. C. Hood. In: Discussions Faraday Soc. No. 24. *Interaction in Ionic Solutions.* Aberdeen: Aberdeen Univ. Press, 1958, p. 87.
16. Rossotti, F. J. C. In: *Modern Coordination Chemistry,* edited by J. Lewis and R. G. Williams. New York: Interscience, 1960, p. 2.

17. RYDBERG, J. *Acta Chem. Scand.* 14: 157, 1960.
18. RYDBERG, J. AND J. C. SULLIVAN. *Acta Chem. Scand.* 13: 2057, 1959.
19. SILLÉN, L. G. *J. Inorg. & Nuclear Chem.* 8: 176, 1958.
20. SONNESON, A. *Acta Chem. Scand.* 12: 1937, 1958.
21. SULLIVAN, J. C. AND J. C. HINDMAN. *J. Am. Chem. Soc.* 74: 6091, 1952.
22. SULLIVAN, J. C., J. RYDBERG AND W. F. MILLER. *Acta Chem. Scand.* 13: 2023, 1959.
23. VAUGHN, P. A., J. H. STURDIVANT AND L. C. PAULING. *J. Am. Chem. Soc.* 72: 5477, 1950.
24. YOUNG, T. F., L. F. MARANVILLE AND H. M. SMITH. In: *The Structure of Electrolyte Solution*, edited by W. J. Hamer. New York: Wiley, 1959, p. 35.

GENERAL DISCUSSION

DR. WILLIAMS: The method of determining formation curves described by Dr. Gryder has been derived independently by us (Tomkinson, J. C. and R. J. P. Williams. *J. Chem. Soc.* p. 2010, 1958). It is satisfying that both of us obtained the same equations. We have tested the method thoroughly in the above paper.

DR. SCHEINBERG: Coming down in the limousine yesterday, Dr. Saltman mentioned a few compounds of metals with ligands that had association constants of the order of 10^{30}. I am completely puzzled as to how one measures the concentrations necessary to arrive at association constants of this magnitude. I don't know that you can do it with electrodes measuring potentials and I am unaware that you can do it with any other method. How does one arrive at association constants with this kind of number attached to them?

DR. GRYDER: I am substituting for Dr. Mansfield Clark and I am sure the comment he would make at this point is that those are just calculation numbers. One measures something that he can measure and calculates the answer. By emf measurements one can fool himself into thinking he is measuring concentration down to a very low value because it is possible to calculate back to the concentration from the potential obtained. The actual mechanism by which the measurement is made may involve other procedures but the calculation numbers are self consistent and as a result the equilibrium constants do make sense if they are interpreted in the proper fashion.

DR. MARTELL (added subsequent to the symposium): It should be pointed out that one cannot, of course, measure equilibria involving extremely low concentrations of one or more species reacting with substances having relatively normal concentrations (10^{-4} – 10^{0} M.). As Dr. Gryder has pointed out, measurements of very high stability constants (or very low dissociation constants) are made by indirect methods. The various techniques available for making such measurements have been reviewed recently (Lewis, J. and R. G. Wilkins, Eds. *Modern Coordination Chemistry*. New York: Interscience, 1960, ch. 1; Chaberek, S. and A. E. Martell. *Organic Sequestering Agents*. New York: Wiley, 1959, ch. 3).

Methods of detecting and controlling metal ion levels

CHARLES N. REILLEY
Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina

While methods of detecting and controlling metal ion levels in aqueous solutions of simple composition have been developed to a reasonable state of perfection, the same cannot be said for biological systems where chelating agents of varying descriptions and chelating powers are abundant and, as yet, incompletely described. Nevertheless, it seems eminently worthwhile to review these methods as they pertain to simple situations in hope that such principles may be extended to biological systems, knowing full well that only through continuous feedthrough of information back and forth between the two systems can real progress be achieved.

METAL BUFFERS

In this chapter the nature of interactions which influence metal ion levels in aqueous systems of reasonably simple composition is discussed and the principles involved in the buffering of such metal ion concentrations by chelating agents are described. A metal ion buffer or "metal buffer" may be considered analogous in operation to the well-known hydrogen ion buffers. In the case of hydrogen ion buffers, an equilibrium exists between the protonated and unprotonated substances, i. e., the conjugated acid and base forms:

conjugate base		conjugate acid	
$H^+ + Ac^-$	$\rightleftarrows$	HAc	(1)
$H^+ + NH_3$	$\rightleftarrows$	NH_4^+	(2)
$H^+ + CO_3^{-2}$	$\rightleftarrows$	HCO_3^-	(3)
$H^+ + HCO_3^-$	$\rightleftarrows$	H_2CO_3	(4)

Notice that in the case of hydrogen ion buffers, the difference in the conjugate base and conjugate acid forms is one hydrogen ion. Systems which contain high concentrations of the conjugate base and conjugate acid are well buffered in respect to the hydrogen ion concentration while systems containing only the conjugate base or conjugate acid are poorly buffered.

In the case of metal ion buffers, the following type of equilibrium are pertinent:

conjugate base		conjugate acid	
$Cu^{+2} + EDTA^{-4}$	$\rightleftarrows$	$Cu\text{-}EDTA^{-2}$	(5)
$Cu^{+2} + Trien$	$\rightleftarrows$	$Cu\text{-}Trien^{+2}$	(6)

Hence, solutions containing large concentrations of complexing agent (conjugate base) and metal complex (conjugate acid) are well buffered in respect to the metal ion. Just as the hydrogen ion concentration depends on the pK_a and on the relative quantities of conjugate base and conjugate acid forms, the metal ion level depends on the formation constant and on the relative quantities of complexing agent and metal complex. The analogy between hydrogen and metal ion buffers can then be seen from the following equations:

Hydrogen ion buffers:

$$K_a = \frac{[H^+][B^-]}{[HB]} \qquad pH = pK_a + \log \frac{[B^-]}{[HB]} \tag{7}$$

Metal ion buffers:

$$K_{MZ} = \frac{[MZ^{n-m}]}{[M^{+n}][Z^{-m}]} \qquad pM = \log K_{MZ} + \log \frac{[Z^{-m}]}{[MZ^{n-m}]} \tag{8}$$

For hydrogen ion buffers, the pH value is numerically equal to the pK_a value when the concentration of the conjugate base and acid forms are equal. Similarly for metal ion buffers the pM is numerically equal to the logarithm of the formation constant when the concentration of the complexing agent and metal complex are equal.

Thus far we have considered complexes which unite in a 1:1 ratio with metal ions. A situation different from that encountered with hydrogen ion buffers occurs when the metal combines with a complexing agent in ratios other than 1:1. For example,

$$Cu^{+2} + 4NH_3 \rightarrow Cu(NH_3)_4^{+2} \tag{9}$$

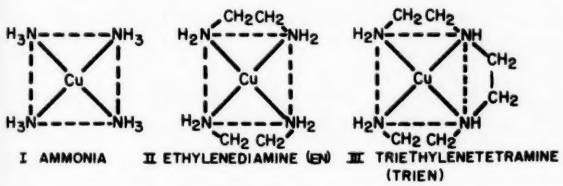
$$K_{Cu(NH_3)_4^{+2}} = \frac{[Cu(NH_3)_4^{+2}]}{[Cu^{+2}][NH_3]^4} \qquad pCu = \log K_{Cu(NH_3)_4^{+2}} + \log \frac{[NH_3]^4}{[Cu(NH_3)_4^{+2}]} \tag{10}$$

For such cases the value of pCu is not necessarily numerically equal to the log of the formation constant of the complex when equal concentrations of complexing agent and metal complex are present. In fact pCu will be numerically equal to log K only when the concentration of each species is one molar. Moreover, dilution of such metal buffer systems will greatly lower the value of pCu, a situation not encountered in hydrogen ion buffers or in metal buffers where only a 1:1 metal-complex is formed

EFFECT OF METAL-COMPLEX RATIO

As pointed out above, the metal-complex ratio has a strong bearing on the buffering ability of a metal-complex system and hence gives rise to behavior unlike that found in hydrogen ion buffers. Because of the importance of these differences they will be discussed in some detail.

Consider the formation of the following complex ions:



whose formation constants are as follows:

Ammonia

$$Cu^{+2} + NH_3 \rightarrow CuNH_3^{+2} \qquad \log K_1 = 4.1$$

$$CuNH_3^{+2} + NH_3 \rightarrow Cu(NH_3)_2^{+2} \qquad \log K_2 = 3.5$$

$$Cu(NH_3)_2^{+2} + NH_3 \rightarrow Cu(NH_4)_3^{+2} \qquad \log K_3 = 2.9$$

$$Cu(NH_3)_3^{+2} + NH_3 \rightarrow Cu(NH_4)_4^{+2} \qquad \log K_4 = 2.1$$

Ethylenediamine (en) (11)

$$Cu^{+2} + en \rightarrow Cu(en)^{+2} \qquad \log K_1 = 10.8$$

$$Cu(en)^{+2} + en \rightarrow Cu(en)_2^{+2} \qquad \log K_2 = 9.3$$

Triethylenetetramine (trien)

$$Cu^{+2} + trien \rightarrow Cu\text{-}trien^{+2} \qquad \log K_1 = 20.1$$

From the values of these formation constants the species present in solution can be readily evaluated. Thus, for example, one may resketch the appropriate equilibria as follows:

$$Cu(NH_3)_4^{+2} \rightleftarrows Cu(NH_3)_3^{+2} \rightleftarrows Cu(NH_3)_2^{+2} \rightleftarrows Cu(NH_3)^{+2} \rightleftarrows Cu$$
$$pNH_3 = \quad 2.1 \qquad\qquad 2.9 \qquad\qquad 3.5 \qquad\qquad 4.1$$

$$Cu(en)_2^{+2} \rightleftarrows Cu(en)^{+2} \rightleftarrows Cu$$
$$p(en) = 9.3 \qquad\qquad 10.8$$

$$Cu(trien)^{+2} \rightleftarrows Cu$$
$$p(trien) = 20.1$$

Thus, if to a solution containing copper ion, ammonia is added in increasing concentration and the uncomplexed residual quantity of ammonia is expressed as pNH_3, the following conclusions can readily be drawn. For values of pNH_3 greater than 4.1, the solution will consist mainly of free copper ions (hydrated). If the ammonia is added until $pNH_3 = 4.1$, the solution will then contain equal quantities of hydrated copper ions and the 1:1 complex, $CuNH_3^{+2}$. If the pNH_3 is decreased to 3.5, the solution will then contain equal quantities of $CuNH_3^{+2}$ and $Cu(NH_3)_2^{+2}$. At values of pNH_3 less than 2.1, the solution will contain $Cu(NH_3)_4^{+2}$ as the predominant form. In the case of ethylenediamine the formation of the 1:1 complex becomes important when p(en) approaches 10.8; and at values of p(en) less than 9.3, the solution contains essentially the 2:1 complex $Cu(en)_2^{+2}$. In the case of trien the complexation of copper commences at a much lower concentration and equal amounts of free and complex copper occur when p(trien) is equal to 20.1.

The effect of complexing agent on the metal ion level (conveniently expressed as pM) can readily be calculated from a knowledge of the stepwise formation constants, K_i, as follows. The total stoichiometric metal concentration (M_t) is equal to the sum of the metal in its various forms,

$$(M_t) = (M) + (MZ) + (MZ_2) + (MZ_3) + \cdots \qquad (12)$$

And upon substituting into equation 12 the relationships similar to those given in equation 11, one then obtains:

$$(M_t) = (M) + (M)(Z)K_1 + (M)(Z)^2(K_1K_2) + (M)(Z)^3(K_1K_2K_3) + \cdots \qquad (13)$$

from which the metal ion concentration, (M), can be obtained

$$(M_t) = (M)(\beta) \quad \text{or} \quad pM = pM_t + \log \beta \qquad (14)$$

where

$$\beta = 1 + (Z)(K_1) + (Z)^2K_1K_2 + (Z)^3K_1K_2K_3 + \cdots \qquad (15)$$

In this way, the metal ion level, pM, can be calculated from the total stoichiometric concentration of metal present, the formation constants for the various complexes, and the values of the ligand concentration, expressed for example as pZ. Figure 1 illustrates the results of such a calculation for a solution containing a total copper concentration of 10^{-2} M where the complexing agents, ammonia, ethylenediamine (en), and triethylenetetramine (trien) are employed. From this figure it is readily seen that small changes in the concentration of ammonia cause larger effects on pCu than of trien (in regions where the four nitrogen ligand atoms are attached to the copper). From this viewpoint metal ion buffers based on polydentate complexing agents are superior to those involving simple ligands. The value of pCu where the concentrations of excess ligand and metal complex are equal, i.e., pZ equals pM_t, follows the order trien > en > NH_3. It is also interesting

to note that at very high concentrations of excess complexing agent p(ligand) approaching -2, the value of pCu is nearly identical for all three cases. Under this situation the population of nitrogen ligand atoms is almost equivalent in the three cases and hence little or no difference in the entropy of "concentration" is encountered. As the concentration of complexing agent decreases, the differing entropies of "concentration" seriously alter the ease of formation of the metal complexes and the effect is greater in the case of the 4:1 complex than in the 2:1 and 1:1 complexes.

It is also interesting to consider Figure 1 in the light of kinetic parameters. Many organic chemicals are synthesized by the use of reagents which may be imagined to form stepwise "complexes" with reacting molecules in achieving the configuration of its activated form. In order for such reactions to occur within a reasonable length of time, it is common and necessary for high concentrations of reactants to be employed. This one may compare as somewhat analogous to the copper-ammonia system. In contrast, reactions involving reagents of a multidentate type, which may be considered for the discussion as analogous to enzymatic reactions, would be more parallel to that of the en and trien situations of Figure 1 and hence could be expected to proceed rapidly even under fairly dilute solution conditions.

INFLUENCE OF VARIOUS FACTORS ON METAL ION LEVELS

Numerous factors govern the metal ion level in addition to the complexing agent, Z, discussed above. For the remaining discussion, matters will be simplified by assuming that Z is multidentate and forms a 1:1 complex with the metal ion of interest, M. Several of the important factors may be summarized by the following equilibrium expression:

$$\begin{array}{ccccccc} \overbrace{\mathrm{M}} & + & \overbrace{\mathrm{Z}} & & \rightleftarrows & \overbrace{\mathrm{MZ}} & \\ + & + & + & + & & + \quad + \quad + & \\ \mathrm{OH^-} & \mathrm{Y} & \mathrm{H^+} & \mathrm{M^*} & & \mathrm{H^+} \quad \mathrm{OH^-} \quad \mathrm{Y} & \\ \Downarrow & \Downarrow & \Downarrow & \Downarrow & & \Downarrow \quad \Downarrow \quad \Downarrow & \\ \mathrm{M_a(OH)_b} & \mathrm{M_aY_b} & \mathrm{H_aZ} & \mathrm{M^*Z} & & \mathrm{H_aMZ} \quad \mathrm{M(OH)_aZ} \quad \mathrm{M(Y)_aZ} & \\ \text{(hydrolysis of metal)} & \text{(complex effect)} & \text{(pH effect)} & \text{(effect of second metal)} & & \text{(effect of metal complex derivatives)} & \end{array} \tag{16}$$

where the charges and the coefficients of the various species have been eliminated in most cases for simplicity. While all of these various competitive equilibria must be considered simultaneously in order to ascertain the metal ion level, each of these effects will be discussed here separately and in an order which facilitates the explanation of each effect.

Effect of pH (2, 4, 13). Equation 16 shows that the hydrogen ion competes with the metal ion, M, for the complexing agent, Z. For this reason, metal ions which form weak complexes may be completely dissociated in acid solution while metal ions which form strong complexes may still be appreciably associated under similar conditions. The exact effect of pH on the metal ion level can be readily calculated from the numerical value of the formation constant of the metal complex and from the acidity constants of the complexing agent. For greatest simplicity, in the final expressions we shall employ the proton formation constants rather than acidity constants, and designate them as follows:

$$\frac{[\mathrm{HZ}]}{[\mathrm{H^+}][\mathrm{Z}]} = \mathrm{k_1}, \qquad \frac{[\mathrm{H_2Z}]}{[\mathrm{H^+}][\mathrm{HZ}]} = \mathrm{k_2}, \cdots \text{etc.} \tag{17}$$

The formation constant for the metal complex is given by equation 8. The residual unmetallized complexing agent exists in solution in several forms, the relative quantity depending upon pH, and its total stoichiometric concentration may be designated as $\mathrm{Z_t}$.

$$[\mathrm{Z_t}] = [\mathrm{Z}] + [\mathrm{HZ}] + [\mathrm{H_2Z}] + \cdots \tag{18}$$

For example, the various species of EDTA which will be prevalent in solution at different pH values may be summarized:

$$\begin{array}{ccccccc} & \overset{\log k_4}{\rightleftarrows} & & \overset{\log k_3}{\rightleftarrows} & & \overset{\log k_2}{\rightleftarrows} & \\ \mathrm{H_4Z} & & \mathrm{H_3Z^{-1}} & & \mathrm{H_2Z^{-2}} & & \\ & \mathrm{pH} = 2 & & 2.76 & & 6.16 & \end{array}$$

$$\begin{array}{ccc} & \overset{\log k_1}{\rightleftarrows} & \\ \mathrm{HZ^{-3}} & & \mathrm{Z^{-4}} \\ & 10.26 & \end{array} \tag{19}$$

Thus, at pH 5 the prevalent form in solution is $\mathrm{H_2Z}$ while at pH 6.16 equal quantities $\mathrm{H_2Z}$ and HZ are present. By substituting in the values for the proton formation constants, expression 18 may be written

$$[\mathrm{Z_t}] = [\mathrm{Z}] + [\mathrm{Z}][\mathrm{H^+}]\mathrm{k_1} + [\mathrm{Z}][\mathrm{H^+}]^2\mathrm{k_1k_2} + [\mathrm{Z}][\mathrm{H^+}]^3\mathrm{k_1k_2k_3} + \cdots \tag{20}$$

and the term [Z] can be factored from this expression to give

$$[\mathrm{Z_t}] = \alpha[\mathrm{Z}] \tag{21}$$

where

$$\alpha = 1 + [\mathrm{H^+}]\mathrm{k_1} + [\mathrm{H^+}]^2\mathrm{k_1k_2} + [\mathrm{H^+}]^3\mathrm{k_1k_2k_3} + \cdots \tag{22}$$

The pH-dependent term, α, is extremely useful in all types of calculations involving complex formation because it facilitates the calculation of the concentration of the completely ionized complex [Z] in terms of the stoichiometric concentration $[\mathrm{Z_t}]$. Substituting equation

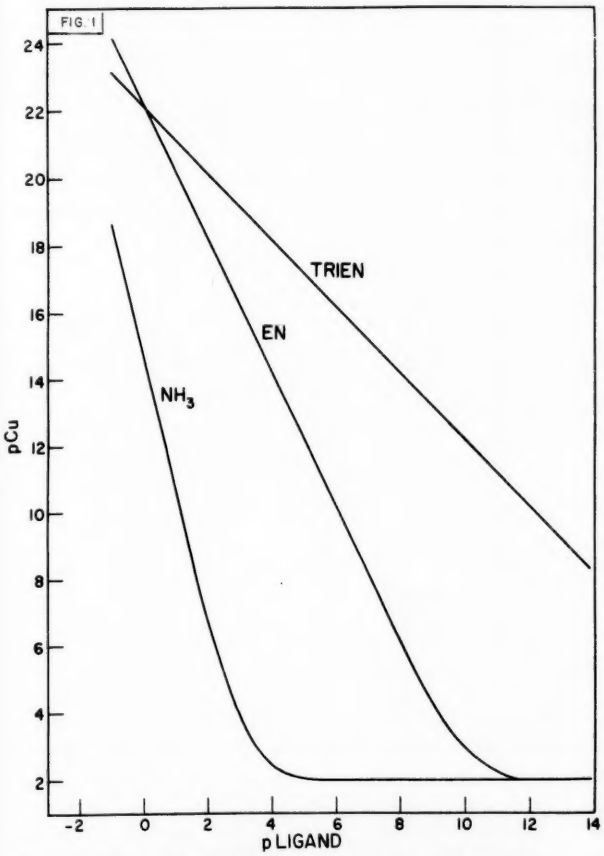


FIG. 1. Effect of metal-ligand on pM-p(ligand) relationship.

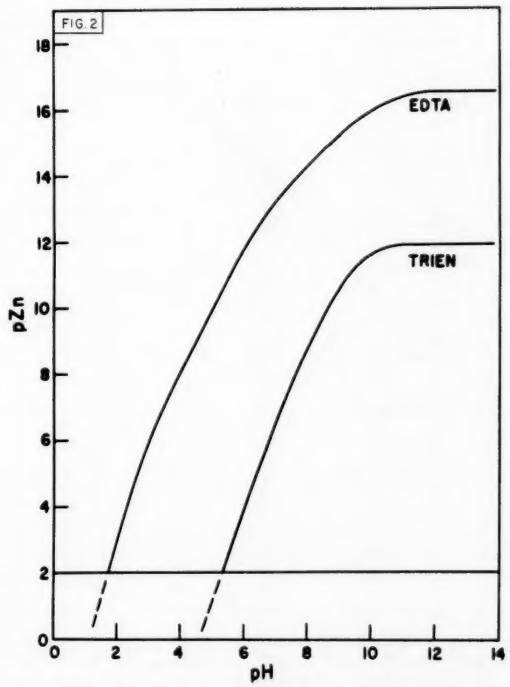


FIG. 2. Effect of pH on metal level of zinc.

21 into equation 8 yields

$$K_{MZ} = \frac{[MZ]\alpha}{[M][Z_t]} \tag{23}$$

which can be rearranged to yield the value of the metal ion level

$$pM = \log K_{MZ} - \log \alpha + \log \frac{[Z_t]}{[MZ]} \tag{24}$$

Figure 2 shows the results for such a calculation for the case of zinc and the complexing agents, EDTA and triethylenetetramine (trien). For this diagram the concentrations of metal complex and excess complexing agent are identical. At pH values greater than 11 the complexing agents exist primarily in their free base form and the value of α is equal to 1. Under these conditions the value of pM is numerically equal to the value of log K_{MZ}, (11.9 for trien and 16.5 for EDTA). The characteristic shape of the pM-pH plot is determined solely by the proton formation constants and is, therefore, usually different for different complexing agents. In the case of EDTA (see equation 19), a break occurs at pH 10.26 and the pZn-pH plot has a slope of 1 in the region of pH between 7 and 10 where the predominant form of EDTA is the monoprotonated form HZ^{-3}. A second break occurs at pH 6.16 and the slope of pZn-pH plot is 2 at pH values between 3 to 6 where the predominant form of EDTA is the diprotonated form, H_2Z^{-2}. The shape of the pZn-pH plot in the case of trien may be similarly interpreted in view of its proton formation constants (log k_1 = 9.92, log k_2 = 9.20, log k_3 = 6.67, log k_4 = 3.32). If the stoichiometric concentration of zinc ion was 10^{-2} molar, one could see from Figure 2 that the trien complex of zinc would be essentially dissociated below pH 5.5.

Figure 3 is a similar plot for various complexing agents with copper and with calcium. Inspection of the figure allows many interesting comparisons, only a few of which will be pointed out here. In alkaline solutions tetren is capable of reducing the concentration of copper ion to the lowest level of any of the complexing agents shown. Yet in acid solutions, i.e., pH 3, tetren is one of the poorest binders of copper. For maximal binding of copper over pH of 3 to 12, a mixture of CyDTA (CDTA) and tetren would be superior to any single complexing

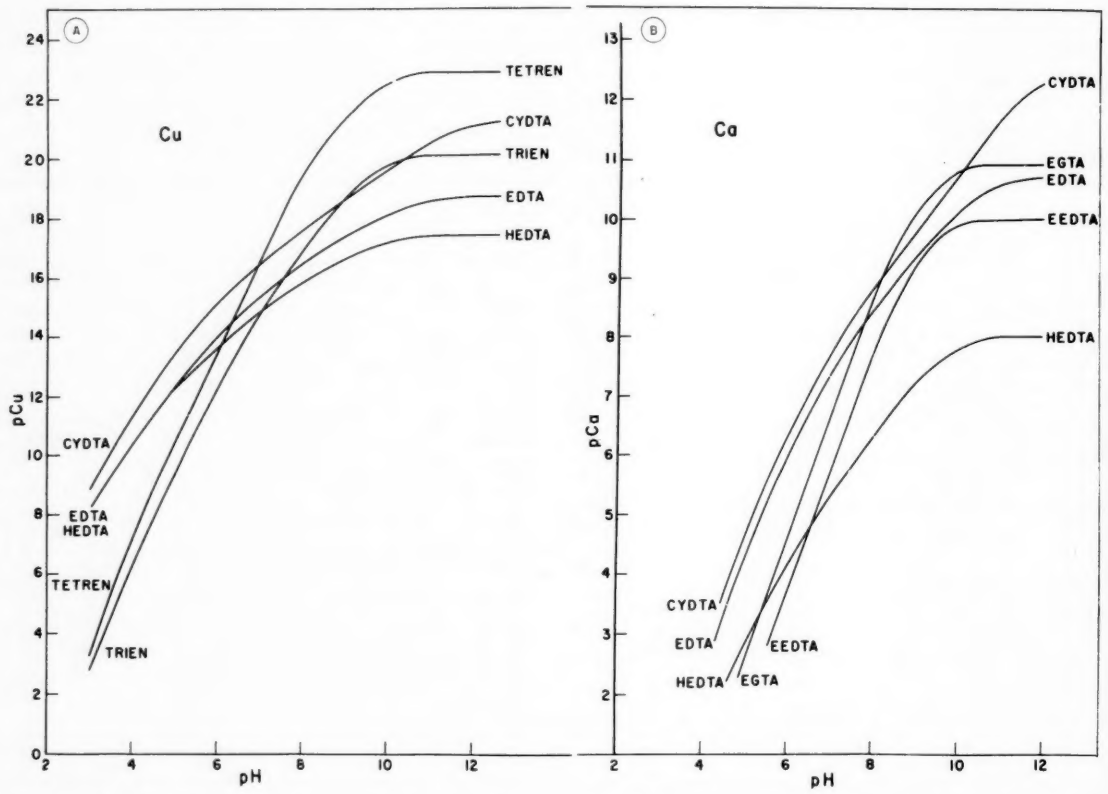


FIG. 3. Effect of pH on metal level of copper (left) and calcium (right) when buffered by different chelating agents. Metal chelate and excess chelate are present in equal concentrations. (CYDTA, 1,2-diaminocyclohexane-N,N,N′,N′,-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEDTA, N-hydroxyethylethylenediaminetriacetic acid; tetren, tetraethylenepentamine; trien, triethylenetetramine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; EEDTA, β,β′-diaminoethylether-tetraacetic acid.)

agent alone. In the case of calcium, the complexing agent, HEDTA, is the poorest shown for alkaline solutions but yet is one of the best for acid solutions.

In summary, evaluation of the various complexing agents based solely on the comparison of the log K values is deceptive, as only after the effects of pH are considered can really justifiable conclusions be made concerning their relative merits.

Effect of Metal Ion Hydrolysis. The pH effects discussed above point to the use of alkaline conditions for most effective metal-binding. The hydrolysis of metal ions under such conditions, however, may eventually raise the pM value sufficiently to overcome the enhancing value of the pH effect. Because of the similar influence of these effects, an optimum pH region will exist for maintaining the metal ion in solution and yet holding the pH value high. The optimum pH region for this will depend upon the stability of the metal complex, the hydrolysis of the metal ion, and the relationship between pH and the log α for the complexing agent. The effects of these competitive equilibria are readily estimated from Figure 4, where the effect of formation of metal hydroxide precipitates and metal-binding with the complexing agent trien is presented as a function of the solution pH. The straight lines marked mercury, copper, cadmium and lead indicate the pM value for the metal ions in question caused by hydrolysis and are given for divalent metal ions by

$$pM = pK_{sp} + 2pH - 28 \qquad (25)$$

where K_{sp} is the solubility constant of the metal hydroxide. The values employed in the calculation are:

Metal Hydroxide	K_{sp}
HgO	3×10^{-26}
$Ni(OH)_2$	1.6×10^{-16}
$Cu(OH)_2$	1.6×10^{-19}
$Zn(OH)_2$	6.3×10^{-17}
$Cd(OH)_2$	1×10^{-13}
$Pb(OH)_2$	8.7×10^{-16}

From the diagram it is readily seen that the hydrolysis of mercury does not interfere except at very high pH values while lead tends to precipitate above pH 11.5. If a 10 fold or 100 fold excess of complexing agent is used, the pH required for precipitation is successively increased as noted in the case of mercury.

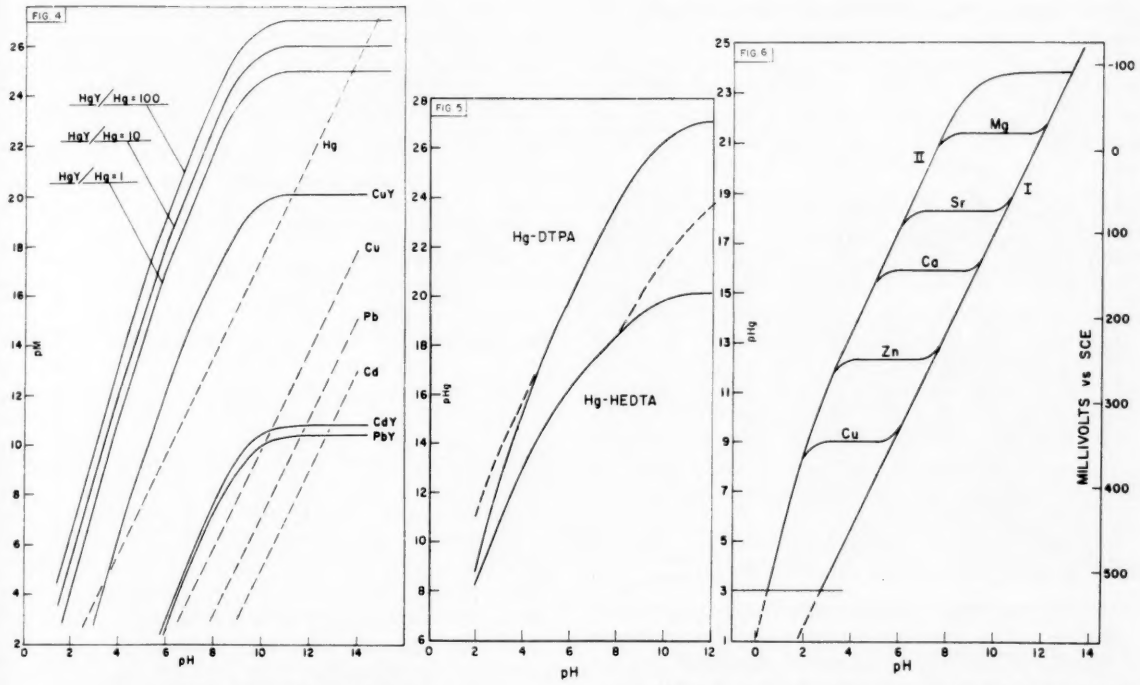


FIG. 4. Effect of metal ion hydrolysis.

FIG. 5. Effect of formation of metal chelate derivatives. (DTPA, diethylenetriaminepentaacetic acid; HEDTA, N-hydroxyethylethylenediaminetriacetic acid.)

FIG. 6. Effect of excess extraneous metal ions (i.e., Ca^{+2}, Zn^{+2}, etc.) on a metal ion level (Hg^{+2}). Hg-EGTA^{-2}, M-EGTA^{-2}, and M^{+2} all 10^{-3} M. (SCE, standard calomel electrode.)

In these cases hydrolysis was assumed to lead only to the insoluble precipitate, but it is well known that hydrolysis usually proceeds stepwise with the formation of one or more intermediate monomeric (i.e., $FeOH^{+2}$) or polymeric (i.e., $Fe_2(OH)_2^{+4}$ and $Bi_6(OH)_6^{+12}$) species [7, 15]. When the total concentration of the metal constituent is very low, the monomeric species are favored.

Effect of Metal Complex Derivatives. The ability of metal complexes to form acid derivatives of the type H_aMZ tends to minimize the rapid decrease in pM as solutions are made acid. The effect usually becomes most predominant near the pH region where the complex commences to dissociate by way of the pH effect. This is illustrated by dashed line in Figure 5 for the case of the mercury complex of DTPA. (This effect is especially prominent with DTPA whose metal complexes form proton derivatives at pH values appreciably higher than that required for dissociation of the complex itself. (1, 16).)

The effect of such proton complexes on pM levels can be calculated by taking into account the value of the proton formation constant,

$$K_{HMZ} = \frac{[HMZ]}{[H^+][MZ]} \tag{26}$$

the fact that the metal complex will exist in both forms,

$$[MZ_t] = [MZ] + [HMZ] \tag{27}$$

which, when inserted into equation 24, yields

$$pM = \log K_{MZ} - \log \alpha + \log \frac{[Z_t]}{[MZ_t]} + \log (1 + K_{HMZ} \times [H^+]) \tag{28}$$

Metal complexes also tend to hydrolyze as the pH increases by forming products of the type $M(OH)_aZ$. The tendency for such hydrolysis varies greatly with the metal under consideration and with the structure of the complexing agent. This topic will be considered in more detail by Dr. Gustafson. This effect also tends to increase the pM level under appropriate pH conditions as illustrated in the case of the mercury-HEDTA complex as shown by the dashed line in Figure 5. In simple cases the hydrolysis of a metal complex can be written as

$$MZ + H_2O \rightarrow M(OH)Z + H^+ \tag{29}$$

whose acidity constant is given by

$$K_{MZ}^{H^+} = \frac{[H^+][M(OH)Z]}{[MZ]} \tag{30}$$

Some values for the mercury complexes of EDTA, HEDTA, and CyDTA are given in Table 1 (6, 8). The tendency for hydrolysis of mercury complexes is appreciably weaker in the case of EGTA (log K_{MZ} = 23.8), DTPA (log K_{MZ} = 27.0), trien (log K_{MZ} = 25.0), and

TABLE 1. *Hydroxo and Ammine Derivatives of Mercury Complexes*

Complex	log K_{MZ}	$-\log K^{H^+}_{HgZ}$	log $K_{Hg(NH_3)Z}$
HEDTA	20.1	8.4	6.1
EDTA	22.1	8.9	6.4
CyDTA	24.4	10.3	5.5

tetren (log K_{MZ} = 27.7). At first there appears to be only a small correlation between the stability of the metal complex and its tendency to hydrolyze. However, for complexes of similar skeletal structures (such as in Table 1), there tends to be a strong correlation between the ease of hydrolysis and the stability of the metal complex where the ease with which a ligand can be removed to allow bonding to an OH^- ion is indirectly related to the overall stability of the complex. For complexes of different skeletal structures, it appears that the tendency for hydrolysis decreases as the number of possible ligand sites increases and as the strain in forming the chelate rings decreases.

The effect of metal chelate hydrolysis can be calculated by taking into account equations 30 and 31,

$$[MZ_t] = [MZ] + [M(OH)Z] \tag{31}$$

which, when combined with equation 24, yields

$$pM = \log K_{MZ} - \log \alpha + \log \frac{[Z_t]}{[MZ_t]} + \log \left(1 + \frac{K^{H^+}_{MZ}}{[H^+]}\right) \tag{32}$$

Some metal chelates also form derivatives of the type MZY_a where Y can be species such as NH_3, Cl^-, CNS^-, pyridine, and occasionally multidentate ligands. (12, 14) Table 1 lists the constants for the formation of ammine derivatives of several mercury chelates where

$$K_{Hg(NH_3)Z} = \frac{[Hg(NH_3)Z]}{[NH_3][HgZ]} \tag{33}$$

Mercury chelates of EGTA, HEDTA, DTPA, and triethylenetetramine and tetraethylenepentamine form only very weak, if any, derivatives with ammonia. Again, for structures of similar skeletal type, the formation of complex derivatives of metal complexes seems to increase as the stability of the metal complex decreases. With metal complexes of a different skeletal structure, this no longer is true. As might be expected, the ease of hydrolysis and the stability of the mixed ammine complexes are somewhat parallel.

The formation of such mixed complexes may be of immense importance in the removal of metal ions in biological systems *via* treatment with complexing agents. For example, even though the mercury-EDTA stability is high, the mercury-EDTA complex itself can react further with basic nitrogen derivatives. It is, therefore, not inconceivable that the metal complex itself can bind to biological substrates (i.e., at amine groups) and hence its removal will be deterred.

Effect of a Second Metal Ion. As can be seen from equation 16, the metal ion level can be considerably altered by the presence of a second metal ion (denoted as M*). This effect will be important only when the second metal ion is present in sufficient concentration to complex any remaining complexing agent and in turn appear in excess. The effect of the second metal ion can be illustrated by the following exchange equilibrium:

$$M^* + MZ \rightleftarrows M^*Z + M \tag{34}$$

whose equilibrium constant is given by

$$\frac{[M][M^*Z]}{[M^*][MZ]} = \frac{K_{M^*Z}}{K_{MZ}} \tag{35}$$

From this, one can solve for the metal ion level

$$pM = \log \frac{K_{MZ}}{K_{M^*Z}} + \log \frac{[M^*Z]}{[M^*][MZ]} \tag{36}$$

Figure 6 illustrates the effect of several metals on the pHg level for the EGTA system (4, 10). As can be seen from equation 36, the value of pHg would be constant and independent of pH provided that the concentration of M*Z, M*, and MZ were constant. Indeed, the figure shows that in the presence of the second metal ion, a pH-independent buffer region is achieved. At lower pH values the pM value decreases because the M*Z dissociates and the curve blends into that for the normal pH effect (line II). At high pH values the pHg level is eventually determined by the hydrolysis of mercuric ion (line I).

It is very important to recognize that concurrent effects may greatly influence the metal ion level which, when present alone, would have little influence (5). For example, the equilibrium for the reaction

$$Ca^{++} + \text{Hg-EGTA} \rightleftarrows \text{Ca-EGTA} + Hg^{++} \tag{37}$$

lies considerably to the left as indicated by the value of the equilibrium constant

$$\frac{K_{Ca\text{-}EGTA}}{K_{Hg\text{-}EGTA}} = \frac{10^{10.9}}{10^{23.8}} = 10^{-12.9} \tag{38}$$

However, in the presence of ammonia, the equilibrium becomes

$$Ca^{++} + \text{Hg-EGTA} + 2NH_3 \rightleftarrows \text{Ca-EGTA} + Hg(NH_3)_2^{++} \tag{39}$$

Because of the rather large stability of the mercury-diammine complex,

$$\frac{[Hg(NH_3)_2^{++}]}{[Hg^{++}][NH_3]^2} = 10^{17.5} \tag{40}$$

The equilibrium constant for equation 39 is

$$\frac{[\text{Ca-EGTA}][Hg(NH_3)_2^{++}]}{[Ca^{++}][\text{Hg-EGTA}][NH_3]^2} = 10^{4.6} \tag{41}$$

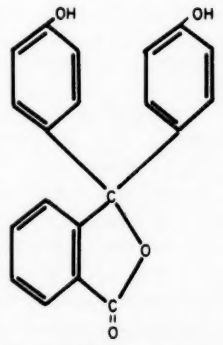
In view of this, it is not surprising that the treatment of mercury poisoning with complexing agents has not yet met with full success.

METHODS OF DETERMINING METAL LEVELS

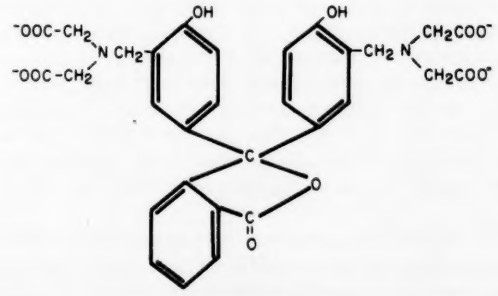
In the above discussion, the metal levels were determined *via* calculation of the pertinent equilibria and their equilibrium constants. Several experimental methods for determining metal levels exist, but they are far from being totally satisfactory. An ideal technique for determining a metal level a) should not shift the position of any equilibria to an extent such that unreliable values of pM result, b) should be specific for a given metal in the presence of its complexes and other metal ions, c) should be applicable over a wide range of pM values, and d) should respond reasonably rapidly. The principles of several techniques with their limitations and possible scope of application are given here.

Metal Indicators (2, 7, 11, 13). Just as it is possible to measure the pH of a sufficiently well buffered solution *via* colorimetric acid-base indicators, it is also possible to measure pM levels *via* metal indicators. This technique will suffer the same difficulties as the colorimetric measurement of pH, i. e., salt effects, protein effects, etc.

A number of metal sensitive dyes have been prepared and are commercially available primarily for use in end point detection in complex titrations. These metal indicators are essentially acid-base indicators to which an additional number of ligand atoms has been attached to yield a structural arrangement whereby they can form multidentate complexes with metal ions. A typical example is the modification of the typical acid-base indicator; phenolphthalein(IV), to give the metal sensitive indicator, phthalein purple(V).



IV PHENOLPHTHALEIN



V PHTHALEIN PURPLE

Because the metallized and unmetallized forms of the indicator have different colors, it is possible to determine pM levels under appropriate conditions. To insure that the metal indicator system will not shift the pM level of the system under study by too large a factor, it must be added in minute quantities. Hence, it is essential that such indicators have large molar extinction coefficients. Fortunately, most of the practical indicators used for complex titrations have molar extinction coefficients of sufficient magnitude so that accurate measurements can be made when the indicator is present in concentrations of only 10^{-5} to 10^{-6} molar.

Just as different acid-base indicators must be used in the colorimetric determination of wide range pH values, it is also necessary to make use of different metal indicators in order to determine the pM level of a solution where a wide pM range occurs. Figure 7 shows the pZn value that can be measured using two different indicators. The solid line indicates the pZn level where the concentration of metallized and unmetallized forms of the metal indicator are equal in concentration, and the dotted region shows the pZn range over which measurements can be made.

Metallochromic indicators themselves are influenced by the same factors shown in equation 16. Because most metallochromic indicators form 1:1 soluble chelates of metal ions, the algebra employed for calculating the various effects is identical in principle with that described previously for the metal complexes. Thus, for MIn

$$pM = \log K_{MIn} - \log \alpha_{In} + \log \frac{[In_t]}{[MIn]} \qquad (42)$$

Similarly, account must be taken of the occasional occurrence of complexes of the type MYIn. Thus, for example, the red zinc-Erio T complex reacts with ammonia to form purple Zn-NH_3-Erio T (11).

In addition the colors of the various protonated forms (e. g., H_2In^-, HIn^{-2}, In^{-3}) of the indicator are important. For Erio T, H_2In^- is pink, HIn^{-2} is blue, and In^{-3} is orange as shown in Figure 8. Hence, measurements for pM levels should preferably be made in pH regions removed from the pK_a of the indicator. The solid line in Figure 8 corresponds to the pMg value at which the indicator is present in equal concentrations in the metallized and unmetallized form. For 1:1 metal indicator complex, the pMg under these conditions is equal to the effective stability constant ($\log K_{MIn} - \log \alpha_{In}$). Thus, at high pH values (greater than 12) where the indicator exists in the non-protonated form ($[In_t] = [In^{-3}]$ and $\alpha_{In} = 1$), the pM value equals the log of stability constant ($\log K_{MIn} = 7.0$ for the magnesium-Erio T complex). As the pH is decreased, a break occurs in the pMg-pH plot corresponding to the pH where the free indicator (In^{-3}) converts to the monohydrogen (HIn^{-2}). As the pH is further decreased, the pM value decreases with a slope of $\Delta pM/\Delta pH$ of 1. At an even lower pH value, where the monohydrogen form (HIn^{-2}) of the indicator converts into the dihydrogen form

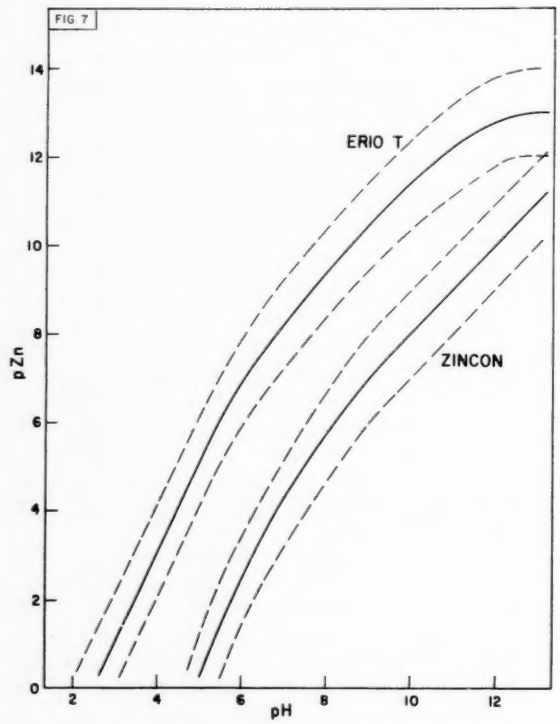


FIG. 7. Colorimetric determination of pZn.

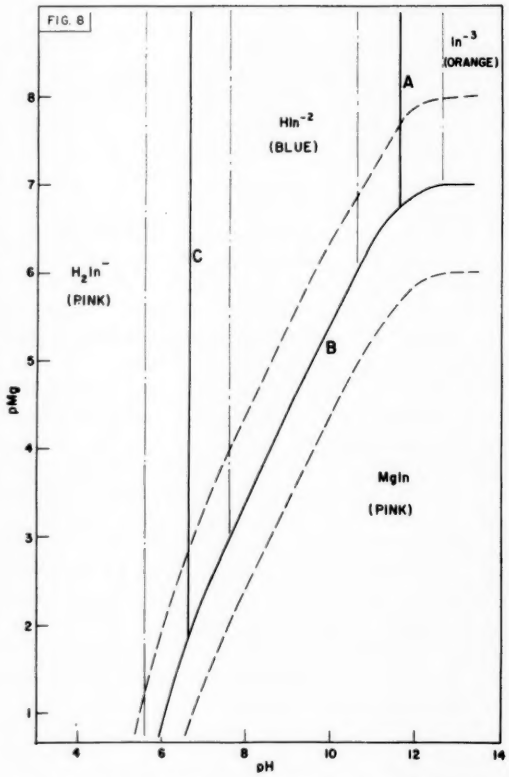


FIG. 8. pMg-pH relationship for the Eriochrome Black T system.

(H_2In^-), a second break occurs at the pM-pH diagram, below which the slope, $\Delta pM/\Delta pH$, becomes 2. These breaks occur, of course, at pH values corresponding to the pK_a values of the indicator; for Erio T, $pK_{a_1} = 6.3$ and $pK_{a_2} = 11.6$.

Hence, to determine the pM level of a substance, a thorough investigation of the indicator and its metal complex is first necessary. Once this is known, it is then possible to use equation 42 to determine the pM level. One simply substitutes the measured ratio of $[In_t]/[MIn]$ into equation 42 along with the appropriate pH (i.e., α) and then solves.

As mentioned above, determination of pM levels *via* indicators will be satisfactory only if the solution is sufficiently well buffered in respect to the metal ion. This means that the addition of 10^{-5} to 10^{-6} molar metal indicator must not cause an appreciable depletion of the buffered constituents, thus changing the pM level.

Another factor that must be taken into account is the kinetics of the various exchange processes. Some metallochromic indicators reach equilibrium only very slowly and this is especially true with certain of the transition metals (i.e., nickel, cobalt, copper, iron).

Lastly, metallochromic indicators, like other chelating agents, are not specific for any one metal ion and hence interfering metal ions must be removed or masked to obtain meaningful results.

Metal Electrodes. In order to determine pM levels potentiometrically, a suitable electrode must be available. Unfortunately, most of the metals exhibit irreversible behavior, and their use is not possible for determining pM levels. A second difficulty is encountered for those reversible electrodes whose metals have high oxidation potentials (i.e., zinc, alkaline earths, and a few others). Their metal electrodes are thermodynamically unstable in aqueous solution and hence evolve hydrogen. Even when their hydrogen overvoltage is decreased by amalgamation, the potentials exhibited are not always reliable. Some metals which are more promising for potentiometric measurement of pM are mercury, copper, bismuth, lead, cadmium, and, in rare cases, zinc.

Undoubtedly, the mercury electrode is one which has been studied rather extensively. Because the principles involved in its application can be extended to other systems, it will be discussed here as a typical example. An example of the application of the mercury electrode is given in Figure 6.

A mercury electrode in contact with a sample solution containing a 1:1 mercury(II) complex and an excess of the complexing agent exhibits a potential corresponding

to the following half cell:

$$Hg \mid HgZ, Z \qquad (43)$$

The potential exhibited by this electrode at 25° C is given by

$$E = E_0 + \frac{0.059}{2} \log [Hg^{++}] \qquad (44)$$

where E_0 is the standard reduction potential of the mercury, mercury(II) system.

Line II represents the increase in the pHg level upon increasing pH. The values of pHg are in excellent agreement with that calculated from stability constants and the known proton formation constants of the complexing agent.

The mercury electrode can also be used as a general pH indicator electrode (9). For this purpose, a mercury electrode is immersed in the solution containing the metal ion, M, whose level is to be determined and a known quantity of mercury(II) chelate, HgZ, as well as a known quantity of metal complex, MZ, is added. Under suitable conditions, this electrode will then exhibit a potential at 25° C given by the following equation:

$$E = E_0 + 0.0296 \log \frac{[M][HgZ][K_{MZ}]}{[MZ][K_{HgZ}]} \qquad (45)$$

where E_0 is again the standard reduction potential of the mercury, mercury(II) system. Because the concentrations of HgZ and MZ as well as the values of K_{MZ} and K_{HgZ} are known for the experiment, equation 45 can be employed for calculating the unknown value of pH. Further, it may be seen that the potential electrode bears a *linear* relationship with pM (provided that the concentrations of HgZ and MZ are constant). Consequently, this electrode may serve as a pM indicator electrode and this feature is particularly significant where suitable electrodes of the first class are not applicable as mentioned above.

For illustrating the principle, solutions were prepared which were 10^{-3} molar in respect to HgZ, MZ, and M, and the potential of the mercury electrode was measured as a function of pH (4). As can be seen from the results given in Figure 6, the horizontal line marked "Sr" corresponds to a pSr = 3. Hence, in the pH region from approximately 6 to 10, this electrode can function as a pSr electrode. For each tenfold change in pSr, the potential would shift 29.6 millivolts. As indicated by equation 46

$$E = E_0 + 0.0296 \log \frac{[HgZ][K_{MZ}]}{[MZ][K_{HgZ}]} - 0.0296\ pM \qquad (46)$$

such a pM electrode can be used to study interaction of metal ion (e.g., Sr) with many complexing agents provided the complexing agent does not react with the metal ion more strongly than with the reference chelate (EGTA).

Other Methods. A novel and promising pM indicator electrode is that recently studied by Gregor and Schonhorn (3). They have described the preparation and properties of a membrane electrode reversible to barium, calcium, and iron(III), which promises to be of use analytically. To prepare such an electrode, multilayers (50 monolayers thick) of the alkaline earth salts of stearic, hexadecyl- and octadecylsulfuric, and hexadecylorthophosphoric acids were deposited on the two "perfect fit" edges of a previously cracked glass slide. The edges could then be rejoined, with the multilayer film now bridging glass to glass (approximately 2500 A). This "crack" constitutes the membrane electrode and allows alkaline earth cations to diffuse through the crack (multilayer membrane) in a direction normal to the axis of orientation of the long-chain acids. When solutions containing different concentrations of barium, calcium, or iron (III) are placed on either side of the crack and SCE electrodes dipped into the solutions, the potential differences measured were found to agree closely with the one calculated for membranes ideally ion-selective for the cation species. The range of ideal performance existed from pH 4 to 10 and ionic strengths from 3×10^{-4} to 15. The choice of 50 layers was made because thicker films tended to be imperfect and thinner ones exhibited too high ohmic impedances. The resistance of a typical membrane (2 cm × 2500 A) ranged from 20 to 60 megohms and thus high impedance instruments were employed as for glass electrode measurements.

The individual electrodes fell into two simple classes: those which were ideally selective and those which did not work at all. The defective electrodes were almost invariably characterized by a lower ohmic resistance of approximately 1 megohm. In view of the fact that the multilayers deposited on the two edges must come together to make a nearly perfect fit (the diameter of the chloride ion is 5 A, so any larger holes through the membrane must be absent), it is remarkable that such a large fraction (approximately 80% after sufficient skill in preparation is achieved) of all membranes prepared were found to be functioning electrodes.

Once assembled, about 0.5 hour of contact with an electrolytic solution was required to secure steady potentials. Thereafter, constant potentials were measured almost instantly after changes in concentration. The electrodes may be stored dry and showed no apparent deterioration after contact with solutions for over 100 hours. Electrodes over one year old were also found perfect.

All of the multilayer membrane electrodes showed an appreciable (1 to 2 mv) asymmetry potential but, unlike glass electrodes, this asymmetry potential was constant throughout the life of the electrode.

Such electrodes can be employed for the determination of barium and calcium salts in the presence of varying concentrations of sodium and potassium salts. They can

also be used to determine the activity of one alkaline earth cation in the presence of high concentrations of other alkaline earth cations.

Because such electrodes can be made reversible to cations such as iron(III) and aluminum(III), they may find eventual use as indicator electrodes in the determination of these and other metal ions by chelometric and other titrations. Analogous multilayer membrane electrodes using positive long-chain molecules may allow potential measurements selective to anions.

The polarographic method, while rapid and straightforward, imposes the severe requirement of a reversible equilibrium in the overall electrode reaction except for the diffusion process. The effect of insufficient rapidly obtained equilibria is that high pM values are obtained, a situation not too uncommon in the case of chelates of high stability. The polarographic method also requires the presence of an electrolyte, frequently a buffered solution, which may interact with free metal ion or metal complex and precludes the determination of accurate pM values under the desired solution conditions. In contrast, potentiometric methods are usually applicable for such systems and do not impose so many restrictions.

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REFERENCES

1. ANDEREGG, G., P. NAGELI, F. MULLER AND G. SCHWARZENBACH. *Helv. Chim. Acta.* 42: 827, 1959.
2. CHABEREK, S. AND A. E. MARTELL. *Organic Sequestering Agents.* New York: Wiley, 1959.
3. GREGOR, H. P. AND H. SCHONHORN. *J. Am. Chem. Soc.* 84: 3911, 1959.
4. HOLLOWAY, J. H. AND C. N. REILLEY. *Anal. Chem.* 32: 249, 1960.
5. MARTIN, A. E. AND C. N. REILLEY. *Anal. Chem.* 31: 992, 1959.
6. REILLEY, C. N. AND W. W. PORTERFIELD. *Anal. Chem.* 28: 443, 1956.
7. RINGBOM, A. *J. Chem. Educ.* 35: 282, 1958.
8. SADEK, F. S. AND C. N. REILLEY. *Anal. Chem.* 31: 494, 1959.
9. SCHMID, R. W. AND C. N. REILLEY. *J. Am. Chem. Soc.* 78: 5513, 1956.
10. SCHMID, R. W. AND C. N. REILLEY. *Anal. Chem.* 29: 264, 1957.
11. SCHMID, R. W. AND C. N. REILLEY. *Anal. Chem.* 31: 887, 1959.
12. SCHWARZENBACH, G. *Helv. Chim. Acta.* 32: 839, 1949.
13. SCHWARZENBACH, G. *Complexometric Titrations*, translated by H. Irving. New York: Interscience, 1957.
14. SCHWARZENBACH, G., H. SENN AND G. ANDEREGG. *Helv. Chim. Acta.* 40: 1886, 1957.
15. SILLÉN, L. G. In: *Treatise on Analytical Chemistry*, Vol. I, Pt. I, edited by I. M. Kolthoff and P. J. Elving. New York: Interscience Encylopedia, Inc., 1959, p. 277.
16. WÄNNINEN, E. *Thesis.* Åbo Academy, Åbo, Finland, 1960.

DISCUSSION

Formation of polynuclear chelates

RICHARD L. GUSTAFSON[1]

Department of Chemistry, Clark University, Worcester, Massachusetts

CONSIDERATION of the role of metal chelate compounds in biological systems is complicated in many cases by the fact that under physiological pH conditions many metal ions and metal chelates undergo disproportionation, hydrolysis and olation reactions:

$$2MA(H_2O)_x \rightleftharpoons MA_2(H_2O)_{x-y} + M[OH]_2(H_2O)_{x-2} + 2H^+ + yH_2O \quad (1)$$

$$MA(H_2O)_x \xrightleftharpoons{K_a} M[OH]\,A(H_2O)_{x-1} + H^+ \quad (2)$$

$$2MA(H_2O)_x \xrightleftharpoons{K_D} (M[OH]A(H_2O)_{x-2})_2 + 2H^+ + 2H_2O \quad (3)$$

$$2M[OH]\,A(H_2O)_{x-1} \xrightleftharpoons{K_d} (M[OH]\,A(H_2O)_{x-2})_2 + 2H_2O \quad (4)$$

Here M represents a metal ion; A, a ligand molecule; and [OH], a coordinated hydroxo group.

In general, the tendency toward hydrolysis of an aqueous metal ion increases with increasing charge and decreasing ionic radius. The degree of hydrolysis may be reduced in many cases by chelating the metal ion with a suitable polydentate ligand. In this way the number of coordination positions available for hydrolysis and olation is reduced and the effective charge of the metal ion is lowered. Investigations of a wide variety of metal chelates have shown (2) that hydrolytic tendencies of partially chelated metal ions increase in the approximate

[1] Present address: Rohm and Haas Company, Philadelphia, Pa.

order Mn(II) < Zn(II) <La(III) <Cu(II) <Th(IV) < Fe(III) < Cr(III) < Zr(IV) < Ti(IV). Chelates of the alkaline earth ions exhibit no hydrolytic tendencies over a wide range of pH. If the coordination requirements of the metal ion are satisfied by a sufficiently large number of donor groups from the organic ligand, hydrolysis and olation reaction generally will not be observed. In some cases, notably those of Fe(III) chelates, displacement of donor groups of various organic ligands by hydroxyl ions is observed under neutral or slightly alkaline pH conditions.

Disproportionation reactions occur in all systems where it is possible to form chelates containing more than one mole of ligand per mole of metal ion. However, the degree to which such reactions occur is dependent upon the relative formation constants, K_{MA} and K_{MA_2}. In the cases of many systems containing equimolar amounts of metal ion and polydentate ligand, the concentrations of species containing two or more ligand molecules bound per metal ion are negligible, representing less than one per cent of the total metal ion concentration. However, there are several systems, such as that of Cu(II)-ethylenediamine, which disproportionate readily in neutral solution.

In the case of the 1:1 chelate of Cu(II)-ethylenediamine, considerable disproportionation takes place simultaneously with hydrolysis because the reactions

$$Cu + A \overset{K_{MA}}{\rightleftharpoons} CuA$$

and

$$CuA + A \overset{K_{MA_2}}{\rightleftharpoons} CuA_2$$

have formation constants which differ by only 1.4 log units. In the case of the 1:1 N-hydroxyethylethylenediamine-Cu(II) chelate where log K_{MA} − log K_{MA_2} = 2.6 much less disproportionation occurs. Hall and Dean (5) have given the following values for the interaction of Cu(II), N-hydroxyethylethylenediamine (HEN) and hydroxyl ion where μ = 0.50 and t = 25.0°:

$$K_{MA} = [CuA]/[Cu][A] = 10^{10.11}$$
$$K_{MA_2} = [CuA_2]/[CuA][A] = 10^{7.51}$$
$$K_a = [Cu[OH]A][H^+]/[CuA] = 10^{-7.21}$$

At μ = 0.10 (KNO_3) and t = 25.0°,

$$K_1 = [HA][H^+]/[H_2A] = 10^{-6.49}$$
$$K_2 = [A][H^+]/[HA] = 10^{-9.52}$$

Also $T_A = [H_2A] + [HA] + [A] + [CuA] + [Cu[OH]A] + 2[CuA_2]$ and $T_M = [Cu] + [CuA] + [Cu[OH]A] + [CuA_2]$ where T_A and T_M represent the stoichiometric concentrations of ligand and metal ion respectively. Solution of the above equations where $T_A = T_M = 5 \times 10^{-3}$ M gives the distribution of species as a function of pH shown in Table 1.

TABLE 1. *Concentrations of Cu(II) and Cu(II)-HEN Species at Various Values of* $-log[H^+]$

$-\log[H^+]$	[CuA]	[CuA_2]	[Cu]	[Cu[OH]A]
6.0	4.29×10^{-3}	2.05×10^{-4}	2.44×10^{-4}	2.64×10^{-4}
7.0	2.91×10^{-3}	1.46×10^{-4}	1.46×10^{-4}	1.80×10^{-3}
8.0	6.88×10^{-4}	3.45×10^{-5}	3.45×10^{-5}	4.24×10^{-3}

TABLE 2. *Hydrolysis and Dimerization Constants of Cu(II) Chelates at 25° in 0.1 M* KNO_3

Ligand	pK_a	log K_d
N,N′-dimethylethylenediamine (4)	8.09	3.8
N,N,N′,N′-tetramethylethylenediamine (4)	8.00	3.9
N-hydroxyethylethylenediamine (3, 4)	7.30	2.2
N,N′-dihydroxyethylethylenediamine (3, 4)	7.15	1.4
α,α′-dipyridyl (4)	7.9	5.0
1,10-phenanthroline (4)	7.8	5.0
1,3-diaminopropane* (3)	7.70	2.6
diethylenetriamine (3)	9.03	—
triethylenetetramine (3)	10.8	—

* No supporting electrolyte employed.

TABLE 3. *Hydrolysis and Olation Constants for Fe(III), Zr(IV) and Th(IV) Chelates at 25°*

Metal	Ligand	pK_a	log K_d
Fe(III)[a, d]	EDTA	7.58	2.95
Fe(III)[a, d]	CDTA	9.32	1.01
Fe(III)[a, d]	HEDTA	4.1	2.4
Zr(IV)[b, e]	EDTA	6.2	3.5
Th(IV)[e]	EDTA (1)	7.04	4.3
Th(IV)[e]	CDTA (1)	7.85	4.3
Th(IV)[e, f]	HEDTA	5.4	5.2

[a] in 1.0 M KCl. [b] in 0.1 M KCl. [e] in 0.1 M KNO_3. [d] R. L. Gustafson and A. E. Martell, unpublished results. [e] B. J. Intorre and A. E. Martell, unpublished results. [f] R. F. Bogucki and A. E. Martell, unpublished results.

In the above calculations, hydrolyzed cupric species as well as chelate species $(Cu[OH]A)_2$ and $Cu[OH]_2A$ have been neglected. It may be shown that lack of inclusion of these species does not affect the results appreciably.

As may be seen in Table 1, the amount of CuA_2 is small, amounting to only 2.9 and 0.8 per cent of T_M at pH 7.0 and 8.0, respectively. Unbound Cu(II) concentrations at these pH values in the above case are such that the solubility product of cupric hydroxide is exceeded. However, in many experiments, including those involving Cu(II)-HEN, no precipitation has been observed. This may be due to supersaturation in the dilute solutions involved.

Since, in addition to metal ions normally present in the human system, many other metals are ingested either accidentally or in the form of therapeutic agents, consideration of a wide variety of metal chelate systems is appropriate. Systems which have been extensively studied in recent years are those of Cu(II), Fe(III), Zr(IV), Th(IV) and UO_2(VI). Many Cu(II) chelates of bidentate ligands (Table 2) have been shown to form binuclear hydroxo chelates. In general, chelates of the least basic ligands dimerize to the greatest extent. This is to be expected since a chelate in which the Cu(II) ion forms a weaker bond with a ligand would have a greater affinity for an additional donor group such as a hydroxyl ion.

In the cases of Cu(II)-diethylenetriamine and Cu(II)-triethylenetetramine, where a third or fourth nitrogen atom is bound to the metal ion, polymerization does not take place, although hydroxo species of both chelates are formed. Chelates of N-hydroxyethylethylenediamine and N,N′-dihydroxyethylethylenediamine hydrolyze readily but polymerize only to a small degree, suggesting that the oxygen atoms of the hydroxyethyl groups are bound to the metal ions to form tridentate and tetradentate chelates.

On the basis of the behavior of the Cu(II)-chelate systems listed in Table 2 it appears that the factors favoring hydrolysis and olation are: *1*) formation of a chelate which is sufficiently stable to prevent precipitation of cupric hydroxide; *2*) high stability of the 1:1 chelate relative to that of the 2:1 chelate; *3*) absence of more than two strongly coordinating donor groups on the ligand.

Recent studies have been carried out concerning the hydrolysis and olation of Fe(III), Zr(IV) and Th(IV) chelates of ethylenediaminetetraacetic acid (EDTA), trans-1,2-diaminocyclohexanetetraacetic acid (CDTA) and N-hydroxyethylethylenediaminetriacetic acid (HEDTA). A summary of hydrolysis and olation constants obtained for these systems is given in Table 3. Here it may be seen that in the cases of Fe(III) chelates even though all six coordination positions of the metal ions may be occupied by donor groups of the organic ligands, there is replacement of the acetate groups of the various ligands by more strongly bound hydroxo groups. Additional unpublished experiments have shown that the driving force in the reaction

$$2\ \mathrm{Fe[OH]EDTA} \rightleftharpoons (\mathrm{Fe[OH]EDTA})_2$$

is a favorable enthalpy change, the entropy change for the reaction actually being negative. Previous results (4) concerning the reaction

$$2\ \mathrm{Cu[OH]A} \rightleftharpoons (\mathrm{Cu[OH]A})_2 + 2\mathrm{H_2O}$$

obtained for a series of cupric diamine chelates, showed that the reaction was favored by a large, positive entropy change, produced by the release of the coordinated water molecules when dimerization occurred.

Comparison of hydrolysis and olation constants for EDTA chelates shows that although the basicity of the chelates increases in the order Zr < Th < Fe, the values of K_d increase in the order Fe < Zr < Th. The lower tendency toward dimer formation of the Zr(IV) chelate relative to that of the more basic Th(IV) chelate may be due to steric hindrance caused by the small size of the central zirconium atom.

Th(IV) and Fe(III) chelates of CDTA show a much more pronounced tendency to hydrolyze than do the corresponding chelates of the less basic ligand, EDTA. However, the equilibrium constants, K_d, for the formation of the binuclear chelates of thorium are identical within experimental error. Bogucki and Martell (1) have suggested that the equivalence of the two dimerization constants is the result of two opposing tendencies: *1*) the greater tendency for dimerization of the Th(IV)-CDTA chelate as a result of the stronger binding strength of its coordinated hydroxo group; *2*) the somewhat greater steric effects for the CDTA chelate which might inhibit the formation of a binuclear species.

The tendency toward hydrolysis of HEDTA chelates is considerably greater than that for the other chelates studied, indicating that a proton is dissociated from the bonding hydroxyethyl group of the ligand. In turn, the bridging atom in the binuclear chelate is probably that of the hydroxyethyl group rather than that of a hydroxo group.

The hydrolysis and olation reactions of metal chelates presumably have their counterpart in the protein bound metal ions of biological systems, provided that some of the coordination positions of the metal ion are occupied by water molecules. It is possible that bridging between protein bound metal ions may account in part for the coiled or folded arrangements of proteins in solution.

REFERENCES

1. Bogucki, R. F. and A. E. Martell. *J. Am. Chem. Soc.* 80: 4170, 1958.
2. Courtney, R. C., R. L. Gustafson, S. Chaberek, Jr. and A. E. Martell. *J. Am. Chem. Soc.* 80: 2121, 1958.
3. Courtney, R. C., R. L. Gustafson, S. Chaberek, Jr. and A. E. Martell. *J. Am. Chem. Soc.* 81: 519, 1959.
4. Gustafson, R. L. and A. E. Martell. *J. Am. Chem. Soc.* 81: 525, 1959.
5. Hall, J. L. and W. E. Dean. *J. Am. Chem. Soc.* 80: 4183, 1958.

DISCUSSION

Some factors governing chelating tendencies and selectivities in the interaction of ligands with metal ions

ARTHUR E. MARTELL[1]

Department of Chemistry, Clark University, Worcester, Massachusetts

TWO OF THE MAIN OBJECTIVES of coordination chemists have been to synthesize chelating agents which have the highest possible affinities toward metal ions and to develop ligands which differ as much as possible. A study of published stabilities, in collections such as the Tables of Stability Constants by Bjerrum, Schwarzenbach, and Sillén (3) reveals that the first of these objectives—the achievement of high stability—has been developed about as far as seems possible with the synthesis and application of ethylenediaminetetraacetic acid and of a number of analogous compounds and derivatives. Four of the most common of such compounds are illustrated by Formulas I–IV.

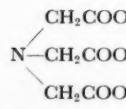
Perhaps the most outstanding success in the development and study of these compounds has been to achieve what classical coordination chemists once considered impossible—the formation of aqueous complexes of the more basic metal ions such as the alkaline earths and the lanthanides with such high thermodynamic stability that these metal ions are largely "deactivated" in aqueous solution. Since the coordination compounds of these metal ions cannot have much covalent character, one visualizes that they are held in a cage of polar groups and of negatively charged groups of the ligand. This concept is in accord with modern theoretical explanations of the stabilities of these coordination compounds—that the complex formation reaction is driven forward by a favorable entropy increase which results from the formation of chelate rings [the chelate effect (4, 6, 7)]. A large part of the stability of these chelates of the more basic metal ions is due to the release of water molecules from both ligand and metal ion, which occurs in the neutralization of charge when the positive metal ion combines with a negative ligand. Thus the greater the degree of charge neutralization that occurs on complex formation, the greater is the stability of the complex. For the most basic metal ions this factor is frequently the most important driving force for the reaction (3, 6).

Schwarzenbach has stated that it does not seem possible to design ligands much more effective than is EDTA in the coordination of metal ions. A survey of the literature indicates that in most cases relatively little is gained when the structure of EDTA is modified in some way that would be expected to increase coordination tendencies. Thus the closing of an alicyclic ring in CDTA (Formula III) has the function of reducing the freedom of rotation of the amino groups in the ligand and thereby directs the coordinating groups toward the metal ion. The increased stability of CDTA chelates, however, is only 2–3 units of log K in most cases, or about equivalent to an additional chelate ring. Increasing the spacing between the amino groups and acetate groups in EDTA largely removes most of the affinity of the ligand for the more basic metal ions. Even an extension of the structure of EDTA to the analogous triamine to give octadentate DTPA (Formula IV), while increasing the affinity for metal ions of very high charge (+3 and +4), does not greatly enhance the affinity of the ligand for Ca(II) and Mg(II) ions.

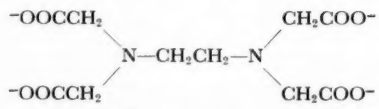
If the structure of EDTA is changed so that the mononegative carboxylate groups are replaced by binegative phosphonate groups, the resulting ligand is even less effective than the parent compound in the binding of many metal ions. Thus stabilities of the more basic metal chelates of ethylenediamine-N,N′-tetramethylenephosphonic acid (EDTMP) (9), shown in Table 1, are so low that they are largely dissociated in aqueous solution except at very high pH. Similar results were recently obtained by Banks and Yerick (2) for the analogous cyclic derivative, the anion of 1,2-diaminocyclohexane-N,N′-tetramethylenephosphonic acid (VI). Presumably, effective binding of metal ions in aqueous solutions is prevented for these ligands by 1) competition with hydrogen ions because of exceptionally high basicity, and 2) strong mutual electrostatic repulsions between the highly charged donor groups which would prevent them from surrounding the metal ion. This effect is somewhat similar to the behavior of a succinic acid derivative which will be described later by Dr. Kroll.

[1] Present address: Dept. of Chemistry, Illinois Institute of Technology, Chicago, Ill.

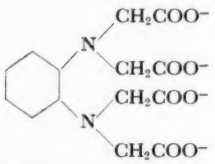
COMMON CHELATING LIGANDS



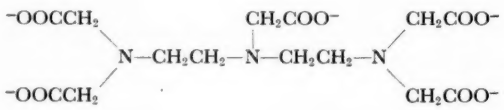
I Nitrilotriacetate (NTA)



II Ethylenediaminetetraacetate (EDTA)

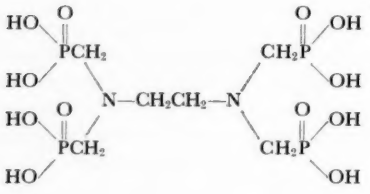


III Trans-1,2-diaminocyclohexane-N,N′-tetraacetate (CDTA)



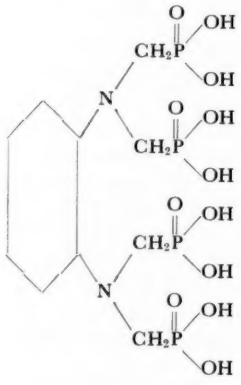
IV Diethylenetriaminepentaacetate (DTPA)

TABLE 1. *Chelating Tendencies of Ligands Containing Phosphonate Groups*



V EDTMP
Ethylenediaminetetramethylenephosphonic Acid

pK_1	1.46	pK_5	6.63
pK_2	2.72	pK_6	7.43
pK_3	5.05	pK_7	9.22
pK_4	6.18	pK_8	10.95



VI CDTMP
Trans-1,2-diaminocyclohexane-N,N′-tetramethylenephosphonic Acid

pK_1	2.40	pK_5	6.97
pK_2	3.70	pK_6	7.69
pK_3	5.32	pK_7	9.39
pK_4	6.46	pK_8	10.89

Metal Ion	Log Stability Constant EDTMP	CDTMP
Ca(II)	6.1	5.4
Mn(II)	9.8	8.4
Cu(II)	>10	9.6
Fe(III)	>10	23.2

From the data in Table 2, it is seen that the stabilities of the chelate compounds of EDTA and of similar compounds are almost entirely an entropy effect for the more basic metal ions. Since the chelate effect and charge-neutralization influence all metal ions in the same way, it is seen that these factors would not contribute to the affinity of the ligand for one metal more than for another. Thus the high stabilities of chelates formed from ligands of this type would not be expected to be associated with any particular degree of selectivity. This principle is illustrated by the data in Table 3. The stability increments which occur when two iminodiacetate groups are replaced by one ethylenediaminetetraacetate ligand are not matched by a uniform increase in selectivity.

Since the chelate effect, an entropy effect, would be expected to operate in a similar fashion toward metal ions of equal charge, one must look for selectivity in the enthalpy of coordination, which is related to the strength of covalent binding between metal ion and ligand. A clue to the type of ligand which is required for differentiating between a transition metal, such as Ni(II), and a more basic metal ion, such as Mg(II), may be obtained from the type of comparison made some time ago by Van Uitert and Fernelius (8), who plotted the differences between the stabilities of Ni(II) and Mg(II) chelates having various types of coordinating groups containing oxygen and nitrogen. The greatest differences were found for the polyamines, and the lowest selectivities (as well as the lowest stabilities) were observed for ligands containing only oxygen atoms. Also, an inspection of equilibrium data (7) shows that the increase in stability of the ethylenediamine complexes of the adjacent transition metals

TABLE 2. *Heats and Entropies of Combination of Aminopolycarboxylate Ligands with Divalent Metal Ions*

Metal Ion	Ligand	$\Delta H°$ kcal/mole	$\Delta S°$ Cal/°C
Mg	NTA	+4	+44
Ca	NTA	0	+31
Ba	NTA	−1	+23
Mn	NTA	−2	+46
Mg	EDTA	−3	+32
Ca	EDTA	−3	+42
Sr	EDTA	−4	+26
Ba	EDTA	−4	+22
Mn	EDTA	−5.2	+41
Co	EDTA	−4.1	+58
Ni	EDTA	−7.6	+55
Cu	EDTA	−8.2	+55
Zn	EDTA	−4.5	+55
Cd	EDTA	−9.1	+38
Pb	EDTA	−13.1	+35

TABLE 3. *Relative Chelating Tendencies and Selectivities of Two Iminodiacetate Ligands and One Ethylenediaminetetraacetate*

Divalent Metal Ion	Iminodiacetate (IMDA) Log K_1K_2	Iminodiacetate (IMDA) ΔLog K_1K_2	Ethylenediaminetetraacetate (EDTA) Log K_1	Ethylenediaminetetraacetate (EDTA) ΔLog K_1	Log K (EDTA) − Log K_1K_2 (IMDA)
Ca	5.4		10.6		5.2
		4.6		5.9	
Cd	10.0		16.5		6.5
		0.1		−2.5	
Fe	10.1		14.0		3.9
		2.1		2.0	
Zn	12.2		16.0		3.8
		0.1		0.2	
Co	12.3		16.2		3.9
		2.3		2.0	
Ni	14.6		18.2		3.6
		1.5		0.3	
Cu	16.1		18.5		2.4

Mn(II) → Cu(II) is greater than for EDTA, in which the diamine is substituted by four acetate groups. Thus it seems that increasing electronegativity of the ligand, and a corresponding increase in ionic character of the coordinate linkages, tend in general to decrease selectivity of the ligand for metals of the first transition series. Conversely, an increase in the tendency of a ligand to form coordinate bonds having greater covalent character leads to greater selectivity. The less electronegative ligands bind the more electronegative metal ions the more strongly and have lower affinities for ions of the more electropositive metals.

The importance of the nature of the donor group in achieving selectivity may be seen in the relative stabilities of metal chelates listed in Table 4, in which one of the oxygen donor atoms of the ligand is successively replaced by a nitrogen atom and by a sulfur atom. It is seen that the spread of stabilities in going from the most basic metal ion, Ba(II), to the least basic, Pb(II), is greatest for the ligand containing a sulfur coordinating group and least for the ligand containing oxygen atoms in the corresponding positions.

Further insight into the nature of the selectivity differences for the transition metals may be obtained by treating stability data according to the method used by George and McClure (5), whereby the relative heats of reaction of the gaseous metal ions, ΔH_L, with the ligand, rather than the normal heats of complex formation from the aquo ion, ΔH_C, are compared. The values of ΔH_L are

TABLE 4. *Chelating Tendencies of Ligands Containing Oxygen, Nitrogen, and Sulfur*

Divalent Metal Ion	Electronegativity	Log Formation Constant: CH_3CH_2OH / CH_2COO^- / N / CH_2COO^-	Log Formation Constant: $CH_2CH_2NH_2$ / CH_2COO^- / N / CH_2COO^-	Log Formation Constant: CH_2COO^- / CH_2COO^- / N / CH_2COO^-	Log Formation Constant: $CH_2CH_2S^-$ / CH_2COO^- / N / CH_2COO^-
Ba	0.97	3.4	3.2	4.8	3.6
Sr	0.99	3.8	3.6	5.0	3.6
Ca	1.04	4.6	4.6	6.4	4.9
Mg	1.23	3.4	4.5	5.4	4.3
Mn	1.60	5.6	7.7	7.4	9.3
Fe	1.64	6.8	9.8	8.8	11.8
Co	1.70	7.9	11.8	10.6	14.7
Ni	1.75	9.3	13.7	11.3	13.7
Cu	1.75	11.9	15.9	12.7	15.9
Cd	1.46	7.5	10.6	9.8	16.7
Zn	1.66	8.3	11.9	10.4	16.7
Hg	(1.9)	5.6	9.7		16.2
Pb	(2.1)	9.4	12.2	11.8	17.0

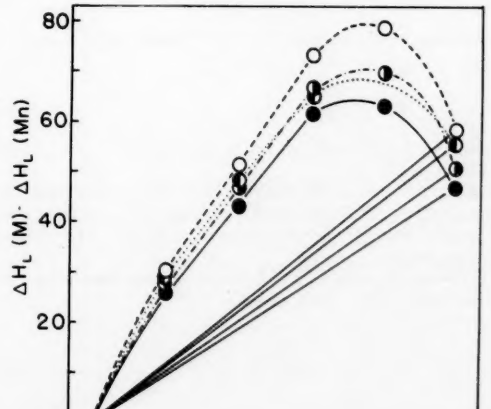


FIG. 1. Relative heats of formation of complexes from gaseous metal ions as a function of the number of 3*d* electrons: ○, triaminotriethylamine; ◐, mercaptoethyliminodiacetate; ◑, nitrilotriacetate; ●, aquo ion.

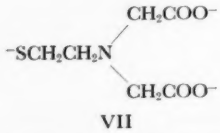
TABLE 5. *Relative Affinities of Ligand Atoms for Various Metal Ions*

More basic metals	
Be(II), Mg(II), Ca(II), Sr(II), Ba(II)	$F^- > Cl^- > Br^- > I^-$
Sc(III), Yt(III), La(III), Ce(III), etc.	O > S(> Se > Te)
Ti(IV), Zr(IV), Hf(IV), Th(IV)	N > P(> As > Sb) F > O > N
Metals of intermediate basicity	
Ga(III), In(III)	$F^- > Cl^- > Br^- > I^-$
Fe(III), Cr(III), Co(III), V(IV)	O > S(> Se > Te)
Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II)	N > P > As > Sb N > O > F
Less basic metals	
Ag(I), Tl(I), Cu(I), Au(I)	$F^- < Cl^- < Br^- < I^-$
Cd(II), Hg(II), Pb(II), Pd(II), Pt(II)	O < S < Se
Tl(III), Au(III)	N < P > As > Sb
Pt(IV)	N ≫ O ≫ F

calculated from the heats of hydration, ΔH_H, the gaseous ions, in accordance with the following scheme:

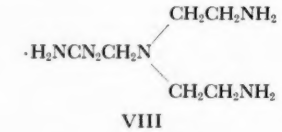
$$M(H_2O)_x^{+2} + yL \rightarrow ML^{+2} + xH_2O \qquad \Delta H_C$$
$$M^{2+}(g) + xH_2O \rightarrow M(H_2O)_x^{+2} \qquad \Delta H_H$$
$$M^{2+}(g) + xL \rightarrow ML^{2+} \qquad \Delta H_L$$

In Fig. 1 the values of ΔH_L, relative to that of Mn, for combination of the series of bivalent metals, Mn, Fe, Co, Ni, Cu, and Zn are plotted against atomic number for three different ligands, NTA, mercaptoethyliminodiacetate (VII) and triaminotriethylamine (VIII). The base lines show the increase in stability



VII

Mercaptoethyliminodiacetate anion



VIII

Triaminotriethylamine

from Mn(II) → Zn(II) that would be expected as the normal result of increased electronegativity of the metal ion, which follows roughly the order of increasing nuclear charge. The vertical distance between the base lines and the corresponding curved plots of $\Delta H_L(M) - \Delta H_L(Mn)$ give a measure of the crystal field stabilization of the metal complexes formed. It is seen that selectivity for Zn(II) relative to Mn(II) is entirely a function of differences in basicity of the ligand since they have the completed shell configurations d^5 and d^{10}, and there is no crystal field contribution to stability. On the other hand, the selectivities for the intermediate metal ions [Fe(II), Co(II), Ni(II), and Cu(II)] result from both differences in basicities of the ligands and from differences in crystal field stabilization of the metal ions by the ligands, the latter effect being more important. The superiority of basic nitrogen groups over oxygen donors in achieving selectivity is readily apparent.

When one attempts to correlate relative affinities of ligand atoms with a wider variety of metal ions, there seems to be an absence of satisfactory guiding principles which agree with experimental evidence. There are many contradictions and reversals even when metal ions and donor atoms are selected from individual groups and series of the periodic system. A careful analysis of the stability data now available does lead to certain generalizations and trends, however, some of which have been pointed out by Ahrland *et al.* (1). Some of these correlations are summarized in condensed form in Table 5, which indicates that the metals may be divided roughly into three groups differing in basicity. For each group a general pattern emerges for the relative affinities of various groups of related ligand atoms. Thus for the more electropositive elements the most electronegative donor atoms are the most effective. For the least electropositive metals, the more polarizable and less electronegative donor atoms show the greatest coordinating tendencies. For the metal ions of intermediate basicity, such as the first transition metal series, mixed orders of donor atom affinities are observed.

REFERENCES

1. Ahrland, S., J. Chatt and N. R. Davies. *Quart. Rev.* (London) 12: 265, 1958.
2. Banks, C. V. and R. E. Yerick. *Anal. Chim. Acta.* 20: 301, 1959.
3. Bjerrum, J., G. Schwarzenbach and L. G. Sillén. *Stability Constants, Part I: Organic Ligands.* London: The Chemical Society, 1957.
4. Chaberek, S. and A. E. Martell. *Organic Sequestering Agents.* New York: Wiley, 1959, chap. 4.
5. George, P. and D. S. McClure. In: *Progress in Inorganic Chemistry,* edited by F. A. Cotton. New York: Interscience, 1959, p. 381.
6. Martell, A. E. *Ann. Rev. Phys. Chem.* 6: 239, 1955.
7. Schwarzenbach, G. *Helv. Chim. Acta* 35: 2344, 1952.
8. Van Uitert, L. G. and W. C. Fernelius. *J. Am. Chem. Soc.* 76: 379, 1954.
9. Westerback, S. and A. E. Martell. *Nature, London* 176: 321, 1956.

GENERAL DISCUSSION

Dr. Williams: Whereas there are certain advantages in the separation of crystal field effects (angular polarization of *d* electrons) from other energy quantities in treating symmetry properties, this separation is apt to be misleading when treating thermodynamic effects. The problem is discussed from a different point of view from that of Dr. Martell and

Dr. Pearson in the *Annual Reports of the Chemical Society*, London, 1960, page 87.

Dr. Martell: As Dr. Williams has suggested, there is an inherent contradiction in the use of ligand-field stabilization and electronegativities to explain thermodynamic properties of transition metal complexes. The ligand-field picture of *d*-orbital stabilization energies is best suited to systems in which the coordinate bonds are primarily ionic. The formation of strong coordinate covalent bonds would certainly influence the energies of the *d* electrons and complicate the simplified interpretation implied by Figure 1. However, I feel that it is helpful to attempt to separate the heats of formation of transition metal complexes (and hydrates) into two easily recognizable effects: the ligand-field stabilization of *d* electrons, and the effect of increasing electronegativity.

Dr. Pearson (added subsequent to the symposium): In reply to Dr. Williams, the straight line one draws from manganese to zinc is intended to represent increasing electronegativity. This, in turn, is created by the effect of a continuously increasing nuclear charge which is screened only partly by the additional electrons added. It is correctly measured by the sum of the two ionization potentials for the process $3d^n\ 4s^2 \rightarrow 3d^n + 2e$, which is a steadily increasing energy. The complication involved in using the ionization potentials of the ground states of the metal atoms is that the special stability of half-filled (d^5) and filled (d^{10}) shells manifests itself. This is well recognized as a quantum-mechanical exchange phenomenon. As such, it does not necessarily have anything to do with electronegativity.

It does produce an effect, of course, if covalent binding is appreciable in which *d* orbitals are used for forming bonds. Systems with d^5 and d^{10} electron configurations will be more reluctant to engage in such covalent bonding. This could be a factor in the drop in hydration energies, etc., exhibited by divalent manganese and zinc. However, the fact that such a good, quantitative explanation of the heats of hydration can be given using optical data to correct for crystal field stabilization suggests, certainly, that covalent effects may be of minor importance in the entire series. At least this argument seems reasonable for covalent bonds using the *d* orbitals of the metal ions.

Metal chelate compounds in biological systems

GUNTHER L. EICHHORN

Gerontology Branch, National Heart Institute, National Institutes of Health, Bethesda, and the Baltimore City Hospitals, Baltimore, Maryland

ONE OF THE MAJOR OBJECTIVES of this conference is the design of chelating agents for the regulation of the concentration of metal ions in biological systems. The success of any attempt to disturb the metal ion balance of a system is obviously dependent upon an adequate understanding of the way in which the metals are bound before the disturbing influence is introduced. Previous speakers have discussed some of the general properties of chelates. The task that has been assigned to me is to review our present understanding of the structure and function of the biological chelates. Unfortunately, our knowledge of both structure and function of biological chelates is exceedingly meager; however, structural information suggests function, and, conversely, the discovery of the function of a chelate can be suggestive of its structure.

The latter consideration leads to the formulation of a fundamental dichotomy of biological chelates. On one side there are those compounds in which the metal ion is present because the metal is needed in a metabolic process. The process may be an enzymatic reaction, such as the enolase reaction, or it may be molecular transport, as in the transport of oxygen by hemoglobin. In either case, the metal ion is designed for the particular process that it mediates. The chelating agent either cannot function at all in the absence of the metal, or else it cannot function as well.

Since metal ions are required for specific metabolic processes, and since the chemical compounds involved in these processes are constantly being synthesized and destroyed, the chelating metals must be delivered to the synthetic site and removed after the chelates have been destroyed. Thus a second group of chelates constitutes the other side of this dichotomy, namely, the chelates that serve to transport and to store metal ions. In these compounds the metal presumably has no function of its own, and is not useful in modifying the properties of the chelating agent. On the other hand, the chelating agent must be specifically designed to bind the metal in transport.

METAL ION TRANSPORT

Let us first consider the compounds in which the chelating agent is designed for the metal ion. The gap between the number of such compounds that must exist to accommodate biologically functional metal ions and the number that have been identified is very large. Nothing is known, for example, of compounds that carry cobalt, manganese, and molybdenum, although each of these metals serves as an enzymatic constituent, and the presence of any of these substances in the free state would wreak havoc in a physiological system. Compounds that store or transport iron, copper, zinc, magnesium, and calcium have been identified.

To illustrate the characteristics of biological compounds that serve in the storage or transport of metals, we shall discuss some of the substances that are involved in the metabolism of iron.

FERRITIN

Probably the most thoroughly investigated of the metal complexes in which the metal does not exert a function is ferritin (38), a protein found primarily in liver, spleen, and bone marrow, but completely absent from blood. This substance is capable of carrying approximately 20% of its weight of iron (32), and is therefore admirably suited for the purpose of iron storage. The iron is present in the +3 oxidation state (39). The binding of metal to protein in ferritin is quite different from simple chelation; rather than providing sites for the attachment of individual atoms of iron, the protein is riddled with vacuoles into which aggregates of phosphorylated ferric hydroxide are packed (38). The iron can be removed from ferritin by reduction with dithionite and complexation with dipyridyl; the resulting apoferritin retains the crystal structure of the iron compound. Apparently the ferric hydroxide has been replaced by water molecules (38).

Experiments with anemic animals have provided ample evidence that the function of ferritin is indeed the storage of iron (19). Of particular interest for the present conference is the fact that no ferritin of low iron content has been obtained from organs that participate in hemoglobin synthesis or decomposition. Michaelis has interpreted this phenomenon as indicating that apoferritin does not lie dormant in the organ to accommodate iron when the latter is released (38). Rather the protein is synthesized whenever the presence of iron requires it, and it is destroyed whenever the iron leaves. Thus ferritin fits the concept of a coordinating agent that is tailored specifically for a specific metal ion. Unfortunately, the

nature of the attachment of iron to apoferritin has not been elucidated.

SIDEROPHILIN

Iron is transported in the plasma by another protein, called siderophilin or transferrin (33), a β-globulin that contains 3.9% sugar (48). The relationship between iron and protein is very different in siderophilin from that in ferritin. The quantity of iron that siderophilin can hold is very much smaller, amounting to just two ferric ions per mole of protein (34). Presumably, therefore, the molecules contain two sites, each of which can hold one iron atom; the specificity of the molecule for iron is demonstrated by the fact that copper, although bound by siderophilin, is held much more loosely and is readily displaced from the molecule by iron (24). The stability of the complex is further emphasized by the inability of the iron, which is in the +3 state (7), to react with dipyridyl in the presence of reducing agents; the iron can, however, be removed by acid (7). Siderophilin, unlike ferritin, is not generally saturated with iron; normally only approximately one-third of the iron sites are occupied (14, 18). Siderophilin, therefore, is considered to be more rapidly available for complexing with iron in case of the production of an excess of iron in an emergency.

OTHER IRON COMPLEXES

Two other substances that are involved in the metabolism of iron are hemosiderin and haptoglobin. Hemosiderin (14, 18), like ferritin, binds large quantities of iron in the form of micelles of ferric hydroxide and is indeed capable of harboring more iron than ferritin, to the extent of 55% of its weight. Hemosiderin appears to be an iron-protein complex into which iron is incorporated when the amount of iron exceeds the ability to synthesize ferritin.

Haptoglobin is a protein that does not bind iron at all but is very firmly bound to hemoglobin; the latter is carried through the plasma as a hemoglobin-haptoglobin complex (33).

Thus there are at least four protein molecules with no other known function than the regulation of the iron content of the body. Two of these, ferritin and hemosiderin, bind large quantities of iron in hydroxide micelles; one of them, siderophilin, binds small quantities of iron atomically dispersed, and one of them, haptoglobin, regulates the transport of hemoglobin, presumably by bonding to the protein portion of the hemoglobin molecule. Thus the complexity of the problem of regulating the iron metabolism can be realized even before any of the compounds in which iron exerts its destined physiological functions has been mentioned. It does not follow that the regulation of iron requires a complete understanding of the structure of all the physiological iron complexes. It may be possible in some instances to mobilize the iron from those compounds in which it is held least tightly, thus achieving regulation at the desired site by indirection. At present, however, not even the relative stabilities of the iron-protein complexes have been determined.

STORAGE AND TRANSPORT OF OTHER METALS

The only other metal whose physiologically inactive (or relatively inactive) complexes have been elucidated structurally to some extent is copper; the metabolism of copper will be discussed by Dr. Scheinberg. Little conclusive information is available on the complexes of zinc, except that the zinc is bound in at least two types of linkage, one much stronger than the other (33, 65). It may well be that several proteins with different affinities for zinc regulate the concentration of this metal; a superficial resemblance to the multiplicity of iron-binding proteins may be noted. The nature of the magnesium- and calcium-binding proteins is even less well understood, although the elucidation of some of these compounds is under way.

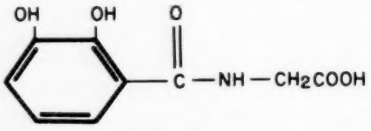
PROTEIN-METAL BINDING

In summary, the only generalization that can be made about the chelating agents that are responsible for the storage and transport of metals is that all those that have been studied are proteins. The linkage of the metals to the proteins is probably via the polar side chains on the proteins, such as the carboxyl, amino, hydroxyl, sulfhydryl, and imidazole groups; peptide linkages also may participate in the coordination when stabilized by chelation; Dr. Martin will discuss this matter further. Most of the presently available information about which groups bind metals in the specific compounds mentioned has come from analogy with model compounds, rather than direct determination. For example, it has been shown that each iron-binding site in avian egg conalbumin contains three hydroxyl groups of tyrosine (66). It has also been shown that the reaction of iron(III) with either siderophilin or conalbumin brings about a similar change in the spectrum of the two compounds; hence it is concluded that tyrosine groups may be implicated in the binding of siderophilin also (33). Because of its ready availability and stability, bovine serum albumin has been used extensively as a model of metal-protein interaction. A recent example of such a study is the finding that copper(II) promotes the dimerization of BSA, but other metals do not (W. W. Everett, personal communication). Such investigations can be valuable guides to the nature of the metal-binding in other proteins, but it is, of course, possible and probable that each protein binds metal ions in its own unique fashion, not excluding the participation of nonprotein components, such as sugars or other prosthetic groups.

Before leaving this group of "nonfunctional" metal complexes, it should be said that any or all of them may be capable of activities other than storage or transport that have yet to be discovered.

Reference might be made also to the studies of Neilands, who has been investigating the ability of microorganisms to synthesize nonprotein chelating agents in

order to improve their capacity for the absorption of iron (15). He has found, for example, that *Bacillus subtilis* is capable of producing 2,3-dihydroxy-benzoylglycine (55):



It is possible that biochemists interested in metal regulation could learn from the microorganisms.

CONTRIBUTION OF METAL IONS TO BIOLOGICAL ACTIVITY: EFFECT OF CHARGE

Having discussed the chelates whose purpose it is to mobilize metal ions in the proper amounts at the proper places for participation in biochemical reactions, we now come to a consideration of those chelates that do have specific biological functions. We shall discuss the various attributes of chelates that make them useful for biological function, and then point out how illustrative biological chelates make use of these attributes.

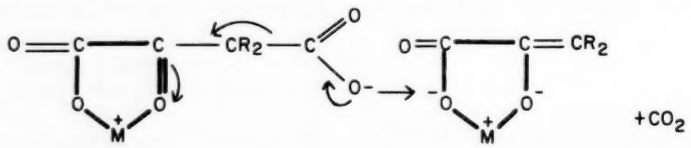
The most important and the most general of these attributes is undoubtedly that the chelation phenomenon involves the interaction of a highly positively charged metal ion with the negative charges or dipoles of the ligands (8). Such charge interaction can be utilized in the transport of electron pair donors through coordination with a metal, as in hemoglobin. The most widespread application of such charge interaction occurs in the participation of metal ions in enzymatic reactions.

METALLOENZYMES

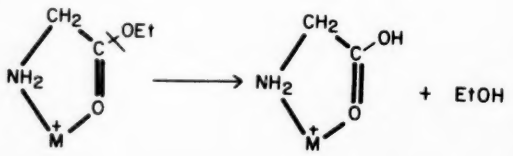
The ligands attached to a metal during the course of an enzymatic reaction may be the enzyme, the substrate, or both enzyme and substrate. Many interesting aspects of the binding of metal ions to enzymes have been discovered by Vallee and Malmström, who will discuss their work later. There are two ways in which interaction with a metal can affect an enzyme molecule: the metal can change the shape of an enzyme to mold it into its functional form, or it can become a part of the active site of the enzyme.

A change in shape of a protein due to reaction with metal ions should be capable of detection through rotatory dispersion measurements, but little advantage has been taken of this technique. Vallee has found that yeast alcohol dehydrogenase yields a Cotton effect in the presence of ortho-phenanthroline (64). Rotatory dispersion measurements have revealed that the presence of a heme group has a profound effect upon the shape of the hemoprotein; this phenomenon will be taken up in detail in a later section.

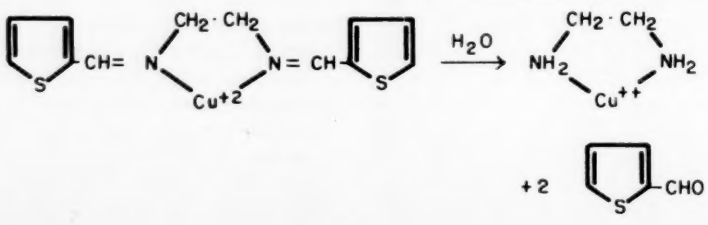
The statement that a metal ion constitutes part of the active site of an enzyme implies that the metal is reacting directly with the substrate molecule. The mechanism by which the metal ion serves to impose the desired change upon the substrate is believed to be a direct consequence of its charge; the highly concentrated positive charge of the generally divalent cation draws electrons toward the metal and therefore away from the substrate. Consequently, a labile bond in the substrate can become more labile and, therefore, more susceptible to cleavage. In a number of cases metal activated enzymatic reactions have been observed when the metal was added to the substrate in the absence of protein. For example, the carboxylase reaction can be catalyzed by a metal ion alone (53, 54, 67). See below:



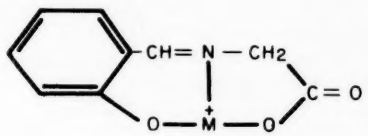
Similarly, the hydrolysis of amino acid esters is mediated by metals (29):



It has been possible to demonstrate, by the use of a model system, that the coordination of a substrate-like molecule with a metal ion can either labilize or stabilize a susceptible bond, depending upon the presence or absence of coordinating ligands in the vicinity of the bond. Thus metal ions hydrolyze the following Schiff base molecule formed from thiophenaldehyde and ethylenediamine (9, 12) as illustrated below:



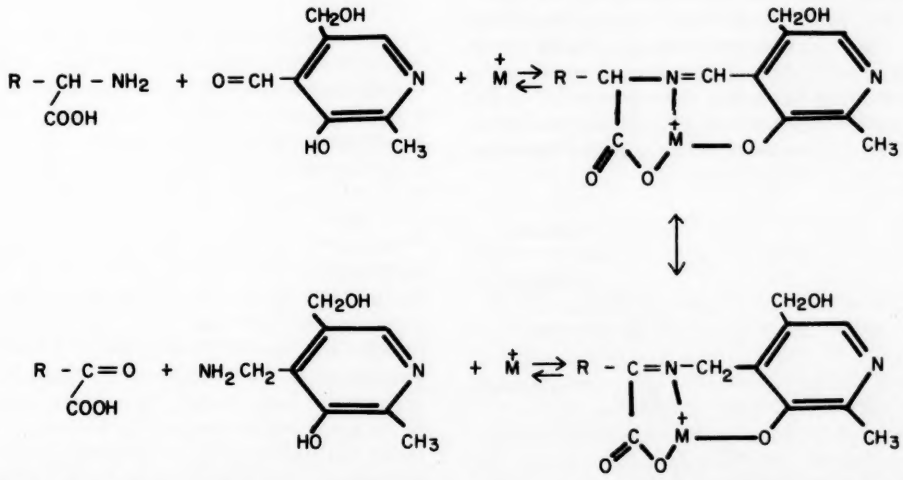
On the other hand, the Schiff base bond between salicylaldehyde and glycine



B

is made more stable by metal coordination (11). Clearly, the charge effect that is responsible for weakening the carbon-nitrogen bond in *A* is also present in *B*; the difference in the behavior of the two molecules lies in the fact that removal of thiophenaldehyde in *A* leaves the chelate intact, whereas the removal of salicylaldehyde from *B* would convert a two-ring chelate into a one-ring chelate. The resultant loss of chelate stabilization more than offsets the effect of metallic charge.

The transamination reaction can be carried out by substituting a metal for the transaminase enzymes. Amino acids and keto acids are interconverted by Schiff base formation, tautomerization, and hydrolysis (5, 10, 50, 51):



Even though it is possible to bring about enzymatic reactions through metal ion "catalysis," the rate of the metal-catalyzed reaction is always orders of magnitude lower than the rate of the enzyme-catalyzed reaction. Consequently, the enzyme-activating metal may, in the absence of enzyme, react so slowly that no reaction can be observed; however, a more reactive metal ion may cause the reaction to occur at a significant rate.

METAL ION SPECIFICITY

The question then arises why it is that the metals that are most efficient in bringing about a reaction nonenzymatically are not also the metal ions that serve as the best activators for the enzymatic process. Dr. Williams has made suggestions along this line (68). I should also like to make a few comments about metal ion specificity.

First of all, an enzyme has to be specific in attacking only one substrate, or else a small number of related substrates; metal ions, however, are exceedingly nonspecific and will coordinate with many molecules that are present in biological systems. Therefore, the most efficient metallic catalyst could promote many undesirable reactions, as well as a given enzymatic process. Furthermore, not only will the reactive metal ions bind nonspecifically to potential substrates but to many functional groups on the protein as well, thus causing the denaturation of the protein. Finally, a most reactive metal could be bound so strongly to the product of the enzymatic reaction that it will not leave the scene of the reaction after the latter has been completed. A catalytic metal ion must be capable of forming a substrate complex that will dissociate as soon as the substrate has undergone transformation.

It should be pointed out in this connection that many of the nonenzymatic models of enzymatic processes, in which metal is added to substrate in the absence of enzyme, are not true catalytic reactions, since stoichiometric amounts of metal ion are frequently employed. The nonenzymatic reactions are models for the activation step of the enzymatic reaction; in the enzymatic reaction the enzyme-metal-substrate complex then decomposes, but in the nonenzymatic reaction the metal-substrate complex frequently remains intact.

In order to test hypotheses about metal ion specificity it is useful to have comparisons of reaction rates with a variety of metal ions acting on the same substrates in both the presence and the absence of enzyme. Few such experiments have been conducted. Speck (52), however, has reported data on the nonenzymatic and the enzymatic decarboxylation of oxalacetic acid.

Examination of the data on the nonenzymatic reaction (Fig. 1) reveals that at low metal concentration (ca. 10^{-3} M) the Irving-Williams series is followed. The rates are in the order (25):

$$Cu > Ni > Zn > Co > Fe > Mn.^{1}$$

[1] The stability of elements of the first transition series varies in the same order as the atomic number, except for zinc.

As the metal concentration is increased, however, the reaction rate for copper passes through a maximum, and at a concentration of $10^{-1.5}$ M, the copper rate is lower than that for any other metal of the first transition series; the other metals are still ordered à la Irving-Williams, or in the order of stability of their complexes. These phenomena can be explained if it is assumed that at high copper concentrations a second mole of copper reacts with the substrate, possibly binding the second carboxyl group, and inhibiting the reaction. It may be noted also that the most active metals below copper are leveling off to a plateau at the highest concentration, whereas the least active metals are still rising at this point. Probably all of the metal ions would eventually inhibit, if it were possible to supply them in high enough concentration.

In the presence of the enzyme, the rate curves (Fig. 2) for all of the metals pass through a maximum in the concentration range being studied, with the exception of the copper rate, which is already on the decline at the lowest concentration, and calcium, magnesium, and barium, which are still on the upgrade at the highest concentration. One effect of the enzyme is, therefore, to decrease the metal concentration at which maximum activity is produced. In the enzymatic reaction the relative activating ability at any given concentration follows no logical order at all, because of the manifold intersections of the curves. But if, instead, one compares the various metals with respect to concentration producing maximum activity, the metals again fall into a more reasonable order, approximating the order of complex stability, although not reproducing its subtleties within the first transition series. The maxima in the enzymatic reaction can be interpreted in the same way as the copper maximum in the nonenzymatic reaction; the excess metal now can react with functional groups on the enzyme, rather than with an additional group on the substrate molecule. As a consequence, manganese activates much better than zinc at $10^{-1.5}$ M concentration, but the order is reversed below $10^{-3.5}$ M.

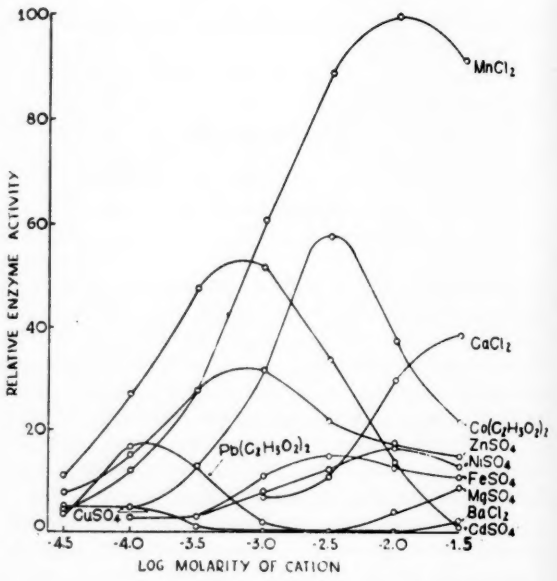


FIG. 2. Effect of metal ions on the enzymatic decarboxylation of oxalacetic acid (52).

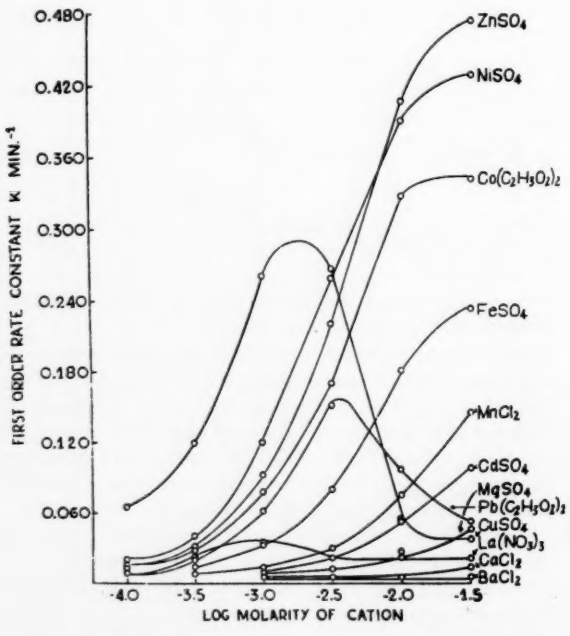


FIG. 1. Effect of metal ions on the nonenzymatic decarboxylation of oxalacetic acid (52).

It may be concluded that both the activation and the inhibition of either the enzymatic or the nonenzymatic reaction follows the Irving-Williams series and that this order becomes confused when both activation and inhibition are going on simultaneously. If this rule can be generalized, it would follow that, when a metal cannot activate but only inhibit, the order of inhibition should be as predicted by the Irving-Williams series. Such an order has indeed been recently confirmed for the hydrolysis of para-nitrophenylacetate by histidines and methylhistidine peptides (36).

EFFECT OF INORGANIC STEREOCHEMISTRY

So far in our consideration of the attributes of chelates that are useful in biological processes we have considered only the charge. A second important characteristic of chelates is that chelation places some very stringent steric requirements upon both metal and ligands. Because metal ions have valence bonds specifically oriented in space, their linkage to one coordinating molecule can change the relative position of the donor atoms within that molecule and coordination with several ligand molecules will determine the relative spatial arrangements of these molecules with respect to each other.

VITAMIN B_{12}

One of the few biological chelates whose structure has been completely elucidated is vitamin B_{12} or cyanocobalamin (2, 23) (Fig. 3). In this molecule the position of the coordinated benzimidazole undoubtedly would be elsewhere, if it were not linked to cobalt, and the spatial position of the cyanide group is determined completely by the fact that the cobalt atom is octahedral.

The biological function of vitamin B_{12} is not understood, although evidence exists that it participates in transmethylation reactions and in protein synthesis. It has been suggested that vitamin B_{12} may mediate the transfer of a methyl group by attaching the methyl group to the cyano nitrogen atom, producing a methyl isonitrile complex (16, 17). Recently, a complex of cobalamin with a polypeptide has been isolated (20). The complex contains no cyanide; presumably, the polypeptide has replaced cyanide in the coordination sphere. When this discovery is considered in light of the participation of the vitamin in protein synthesis, it seems possible that the cyanide group may be incorporated into protein as a peptide linkage and that peptide linkages may, in turn, be converted into cyanide on the surface of the vitamin. Such a hypothesis is made more reasonable by the consideration that any biological activity of the vitamin probably resides on the cobalt, that manifestation of the activity involves removal of the cyanide, and that it is inconceivable that the cyanide could be removed intact. If such a process does indeed occur, the relative positions of the cobalt atom and the propionamide side chains could be an important factor in determining the configuration of the protein.

A consideration of the structural features of the vitamin B_{12} molecule demonstrates how the stereochemistry of metals is one of the factors involved in the choice of a metal for a specific function; copper would not work in vitamin B_{12}, because it will form only four strong coplanar bonds, and the benzimidazole and cyanide groups

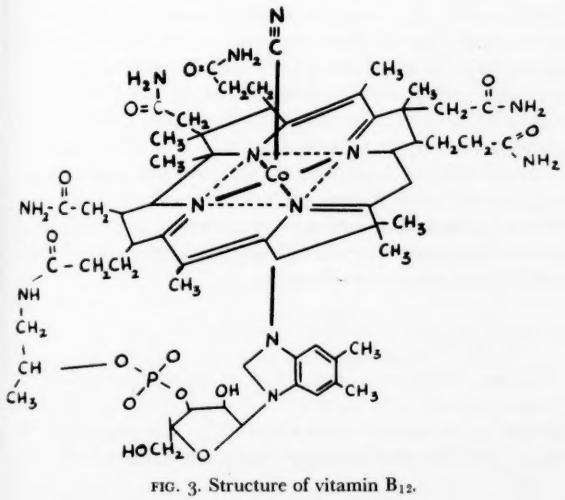


FIG. 3. Structure of vitamin B_{12}.

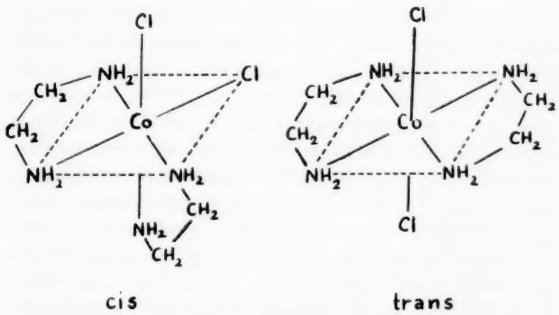


FIG. 4. Geometrical isomers of $[Co(en)_2Cl_2]^+$.

would therefore be only weakly attached and held much farther apart.

EFFECT OF ISOMERISM

A consequence of the stereochemical properties of chelates is that these substances are capable, in many instances, of exhibiting geometrical and optical isomerism. Geometrical isomerism is illustrated by the *cis* and *trans* forms of dichlorobis (ethylenediamine) cobalt(III) ion (Fig. 4).

The chemical properties of these geometrical isomers are very different, as might be expected. The two chlorine atoms in the *cis* complex can be replaced by reaction with a chelating agent such as ethylenediamine, dipyridyl, or ortho-phenanthroline. These chelating agents do not react with the *trans* compound, at least not without isomerization to the *cis* form, since the coordinating groups are sterically prevented from contacting the two *trans* positions.

The relevance of geometrical isomerism to biologically important molecules can be illustrated by the following two examples:

In the vitamin B_{12} molecule just discussed, as well as in the hemoproteins, the cobalt atom, or the iron atom, is attached to four coplanar nitrogens. These coordination positions cannot be substituted without completely removing the metal from the complex. The only substitutable coordination positions are therefore *trans*, and for this reason vitamin B_{12} and the hemoproteins are incapable of any additional chelation and do not react with chelating agents.

The second illustration involves the finding that the zinc in yeast alcohol dehydrogenase (22) and the iron in succinic dehydrogenase (49) will chelate with orthophenanthroline without removing the metal from the enzyme. These experiments imply strongly that the metals in these two enzymes are bound to the protein in such a way as to leave two *cis* positions vacant as indicated in the model illustrated in Figure 5 and are in agreement with the supposition that the substrates of these enzymes are capable of similar chelation.

In addition to the geometrical isomerism just described, chelates are capable of optical isomerism, as illustrated by the D and L forms of *cis* dichlorobis (ethylenediamine) cobalt(III) ion (Fig. 6).

When an asymmetric molecule is produced as a consequence of chelation, the chelated metal is said to be asymmetric. If the chelating agent is itself also asymmetric, coordination with a metal will create a molecule with several asymmetric centers. Frequently a metal in a given optically isomeric configuration will react preferentially with one of the optical isomers of a coordinating agent. Thus, the reaction of cobalt with racemic cyclopentanediamine (26) results in the formation of the tris-(l-cyclopentanediamine)-D-cobalt and the tris-(d-cyclopentanediamine)-L-cobalt (Fig. 7); no isomers in which the d-chelating agent is bound to D-cobalt, or the l-chelating agent to L-cobalt, are formed.

It is conceivable that the stereospecificity of enzymatic reactions may in some instances be a manifestation of the same phenomenon. Let us consider, for example, the action of the metal-activated glycyl-L-leucine dipeptidase. This enzyme does not split glycine-D-leucine at all (6). Since the metal is already attached to an optically active protein molecule, the specificity of the enzyme may be due to the preferential coordination of the metal-enzyme complex with glycyl-L-leucine, rather than with its optical antipode.

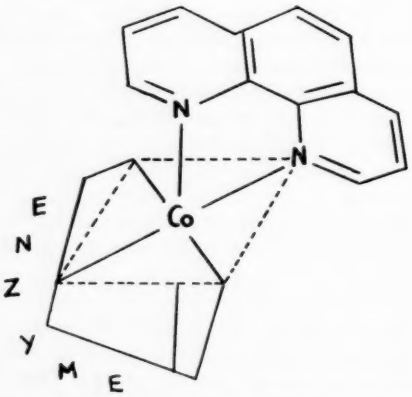


FIG. 5. Structure of enzyme-metal-ortho-phenanthroline complex.

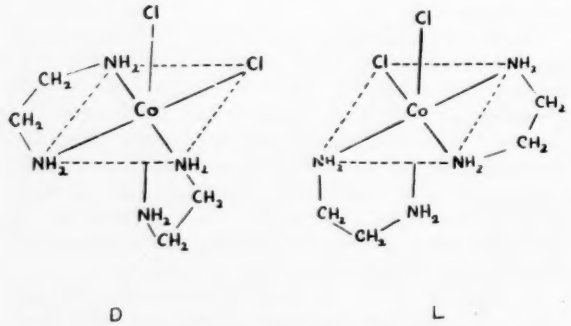


FIG. 6. Optical isomers of *cis* $[Co(en)_2Cl_2]^+$.

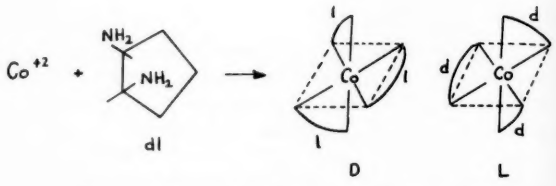


FIG. 7. Reaction of racemic cyclopentanediamine with cobalt.

ELECTRON TRANSFER

Another characteristic of chelates that finds extensive application in biology is their oxidation-reduction potential. Through a proper choice of metal and coordinating agent, remarkable ranges of redox potential are available; the redox potential of a given metal can, in fact, be made to vary at will by slight changes in its coordination sphere.

The metal ions that have been implicated in biological electron transfer are copper (e.g. in the phenol oxidases) (30, 31), molybdenum (in xanthine oxidase (3) and nitrate reductase (40, 41)), and iron, the most predominant of the metals that engages in this activity. Other metals are part of electron transfer systems, but they do not themselves gain or lose electrons in the process.

The most carefully studied group of biological electron transferring molecules is the cytochrome system. The function of the cytochrome system is to provide for a gradual transmission of the oxidizing power of molecular oxygen to the substrates. Instead of one oxidizing agent with a potential so high that no specificity in oxidation could be achieved, the electrons are passed along a chain of compounds of increasingly low potential, thus substituting several low potential steps for one impossibly high potential step. The members of the cytochrome system are hemoproteins. Some of their potentials were first determined by Ball, as follows (1): cytochrome a, -0.29 volts; cytochrome c, -0.27 volts; cytochrome b, $+0.04$ volts. (Potentials of other cytochromes have been recorded by Paul (43).) This variation of redox potential illustrates the range of potentials available from the same metal ion attached to very similar coordinating agents. The hemoproteins are, indeed, an exceedingly adaptable group of biological chelates, in which the same basic complex can be modified by the protein molecule to serve as election transfer agent, or in a variety of other functions. This adaptability of the hemoprotein structure possibly accounts for its evolutionary selection. A discussion of some of the structural aspects of the hemoproteins is very revealing in relating the structure of biological chelates to their function.

THE HEMOPROTEINS

When a metal ion is attached to an enzyme or other protein without benefit of a prosthetic group, it is rather difficult to determine the nature of the ligands, since the metal with its surroundings intact cannot generally be

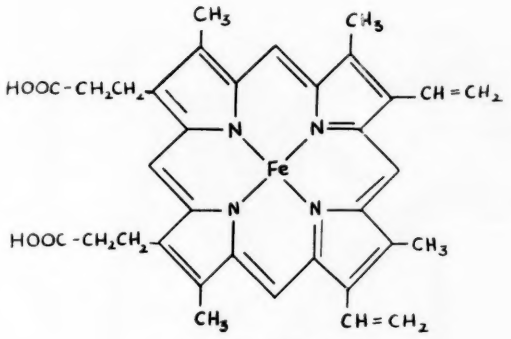


FIG. 8. Structure of heme.

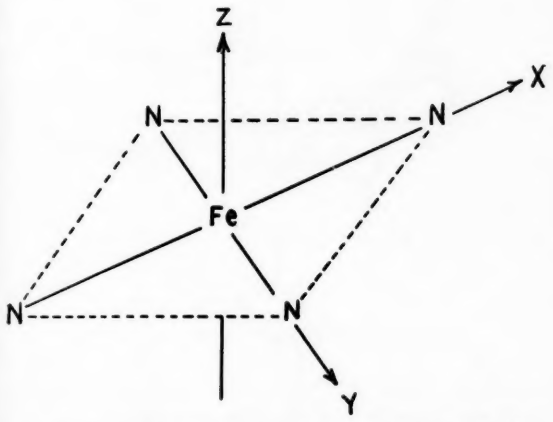


FIG. 9. Geometrical configuration of the covalences of iron (8).

dissociated from the protein without breaking the bonds between the metal and the ligands. It is for this reason that the structure of the hemoproteins and related compounds is understood so much better than the structure of any other biological metal chelates.

All of these compounds are complexes of porphyrins, of which the most common, protoporphyrin (13), is depicted in Figure 8. The iron(II) complex of protoporphyrin is called heme, and the combination of heme with protein is a hemoprotein. To this class of compounds belong hemoglobin, myoglobin, peroxidase, catalase, and the cytochromes.[2]

The active site of all hemoproteins is the iron atom; it is apparent that the iron atom must be modified in a spectacular manner in order to accomplish the variety of functions of these compounds. We can best understand these modifications of the iron atom by reference to the crystal field theory which Dr. Pearson has already introduced.

In all of the hemoproteins four of the coordination positions of the iron atom are taken up by the nitrogen atoms of the porphyrin; the two additional iron bonds are on a perpendicular to the plane of the porphyrin. These spatial relationships can be related most readily to the electronic configuration of iron by superimposing the octahedrally disposed iron bonds upon the Cartesian coordinate system, as in Figure 9 (8).

There are six valence electrons in iron(II) and five in iron (III), all in the five orbitals of the 3-*d* shell. The spatial orientation of the electrons in each of these five orbitals is shown in Figure 10 in the same coordinate system as that introduced for the octahedral iron atom. The d_{xz} and the d_{yz} orbitals are not shown, since their shape is identical with the shape of the d_{xy} orbital, but they lie in the *xz* and *yz* planes instead of the *xy* plane. Each orbital is capable of accommodating two electrons, but since these tend to repel each other, ordinarily each orbital is singly occupied before any becomes doubly occupied.

However, this repulsion of the electrons within an orbital can be overcome by the greater repulsion of the iron electrons and the electrons of the ligands. It will be noted that the d_{z^2} and the $d_{x^2-y^2}$ electrons point directly toward the ligands, whereas the d_{xy}, d_{xz}, and d_{yz} electrons are directed in such a way as not to interfere with the ligands at all. Therefore, the first two orbitals in an octahedral field are more highly energetic and are called the e_g orbitals, whereas the latter three orbitals are less ener-

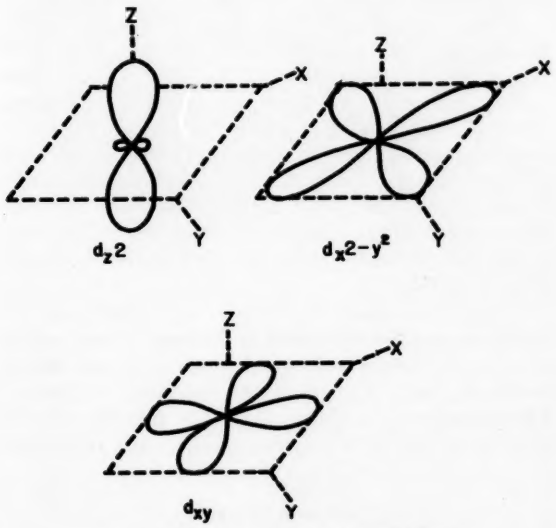


FIG. 10. Spatial orientation of *d*-orbitals (8).

TABLE 1. *Possible Electronic Configurations of Iron (8)*

	Fe(III)		Fe(II)	
	Weak	Strong	Weak	Strong
e_g	×	—	×	—
	×	—	×	—
t_{2g}	×	×	×	××
	×	××	×	××
	×	××	××	××

[2] Some of the cytochromes, e.g. cytochrome a and cytochrome a_3, contain porphyrins other than protoporphyrin (43).

getic and are called the t_{2g} orbitals. When the ligands are held so strongly to the iron atom that the repulsion of the ligand electrons and the iron electrons becomes greater than the repulsion of two electrons in an iron orbital, then the less energetic, i.e. the t_{2g} orbitals, are filled first, before any electrons go into the e_g orbitals; such complexes are strong field, or low spin, complexes. When, on the other hand, the ligands are held relatively weakly, the repulsion between ligand electrons and iron electrons is not so great as the repulsion between the iron electrons in the same orbital, and each orbital becomes singly occupied before any are filled with an electron pair, regardless of whether the orbitals are e_g or t_{2g}; such complexes are weak field, or high spin, complexes. Table 1 compares the electronic configuration of iron(II) and (III) in weak and strong field complexes.

It will now be seen from a comparison of cytochrome c, catalase, peroxidase, myoglobin, and hemoglobin that the electronic configuration assumed by a hemoprotein is closely related to its biological structure and function.

CYTOCHROME C

The amino acid sequence of cytochrome c now has been completely worked out by Margoliash (personal communication). The partial structure of the molecule reproduced in Figure 11 includes that portion of the sequence (61–63) in the vicinity of the points at which the cysteine residues are joined to the vinyl side chains of the porphyrin by addition of two sulfhydryl groups across the vinyl double bonds (21, 56, 57). This strong covalent binding of the protein to two side chains is partly responsible for the extreme stability of the cytochrome c molecule. This stability is enhanced further by coordination of the iron to the protein through two functional groups, which have been represented as imidazole groups (37, 58) although their nature has not been definitely established.

It might be anticipated that this tightly knit structure of cytochrome c would result in the formation of coordinate bonds strong enough to bring about electron pairing in the t_{2g} level. Such is indeed the case, as magnetic measurements have shown (59). The function of cytochrome c, as of all the cytochromes, is to transfer elec-

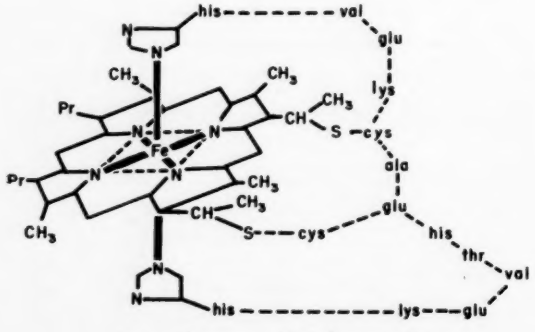


FIG. 11. Structure of cytochrome c (8).

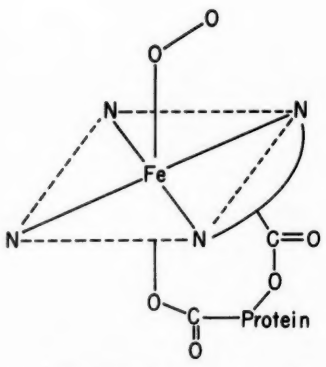


FIG. 12. Ligand attachment in peroxidase (8).

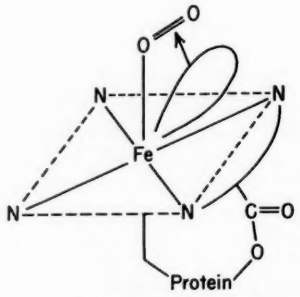


FIG. 13. Ligand attachment in hemoglobin (8).

trons only, rather than to act upon any other molecule. Such activity calls for a structure of maximum stability, in which all of the coordination positions on the iron remain intact at all times. Thus the functional need for stability produces the structural requirement of a strong field complex.

CATALASE AND PEROXIDASE

Although the heme component of catalase and peroxidase is exactly the same as that of cytochrome, the requirements for coordination in the z-direction are very different. In these compounds only one of the z bonds is available for coordination with protein, the sixth coordination bond is reserved for the attachment of peroxide substrate (4) (Fig. 12) and is continually made and broken, when the enzyme exerts its activity. Thus, unlike cytochrome c, catalase and peroxidase require a degree of lability. As predicted, this functional requirement confers the weak field electronic configuration upon the iron atom in these complexes.

MYOGLOBIN AND HEMOGLOBIN

The biological function of myoglobin and hemoglobin is to store and to transport molecular oxygen by coordination with the iron atoms in these compounds. These molecules thus resemble catalase and peroxidase in that the iron atom must be free to coordinate with an extramolecular ligand, in this case molecular oxygen. Accord-

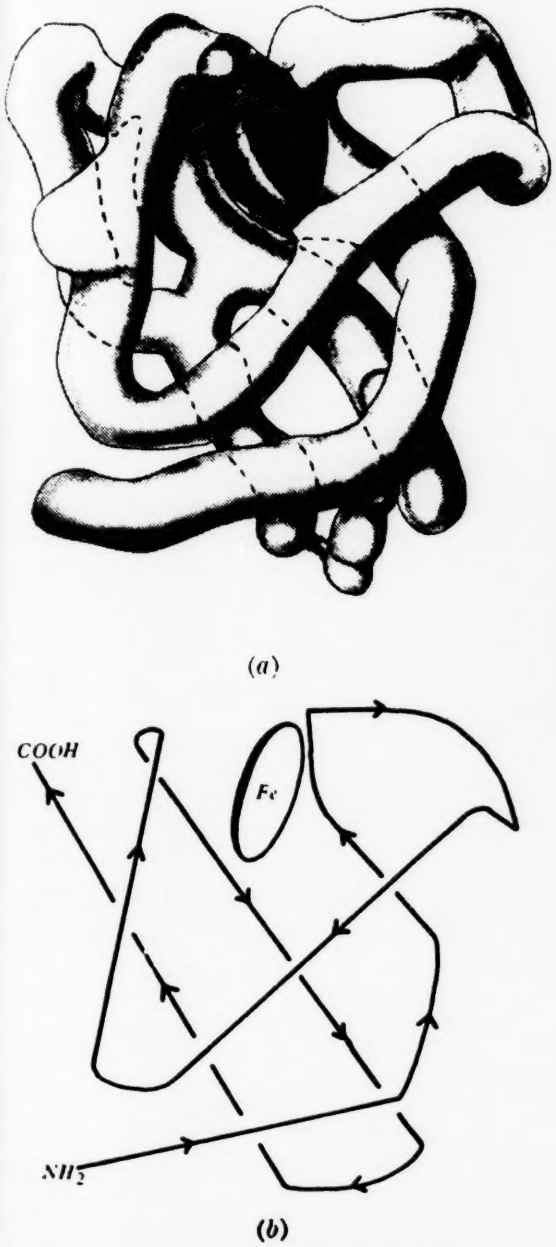


FIG. 14. Structure of myoglobin (28).

ingly, the iron is in the high spin condition (44). However, when the oxygen molecule is bound to the iron, the iron electrons become paired; apparently, the binding to the molecular oxygen increases the ligand field strength enough to overcome the reluctance of electrons to pair in the same orbital. The extra strength is gained by the interaction of a π bond in the oxygen molecule with a t_{2g} orbital of the iron, as illustrated in Figure 13.

Thus the electronic configuration of the iron in the various hemoproteins reflects the biological function they are made to serve.

Recently, myoglobin and hemoglobin have become the first large biomolecules to have their tertiary structure completely worked out. Kendrew and co-workers (27, 28) have shown by X-ray analysis that the protein consists of one strand, helical throughout most of its length, that winds around (see Fig. 14) in such a way so as to hold the heme group close to the surface of the molecule, with one side attached to the protein strand and the other side far enough away from protein so as to accommodate either an oxygen or a water molecule. Similar X-ray studies by Perutz and co-workers (45) have revealed that the hemoglobin molecule consists of four protein strands, each of which resembles the one myo-

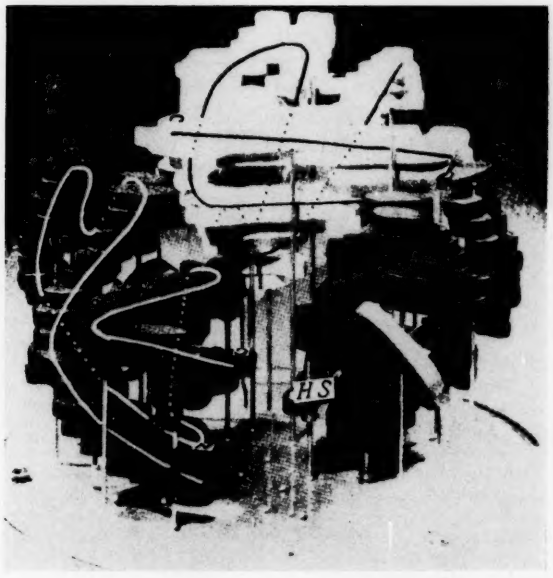


FIG. 15. Partial structure of hemoglobin (45).

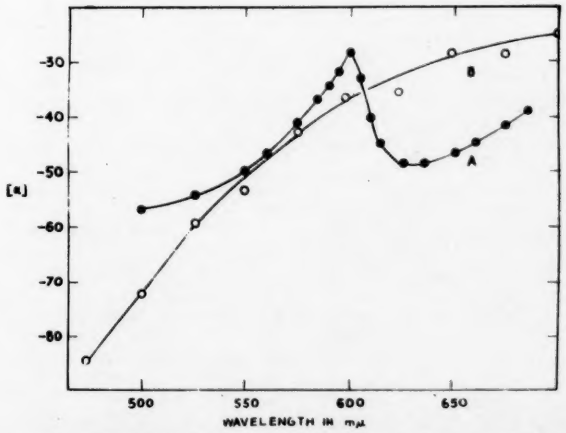


FIG. 16. Rotatory dispersion of catalase: A, intact molecule; B, reconstituted molecule.

globin strand in the manner of its folding and is bound to the heme group in a similar fashion. There are two pairs of identical strands. Figure 15 demonstrates a model of the molecule partly assembled, with three of the strands shown; the fourth strand would fit in the front of the picture, at the level of the center strand, so that the four strands together are tetrahedrally disposed toward each other. The four strands seem to be made to fit each other and to produce a tightly knit spherical structure.

FIT OF THE HEME IN THE HEMOPROTEIN MOLECULE

The work of Kendrew and Perutz has demonstrated how well nature has designed these chelating agents so that they can hold the hemes in the appropriate position for carrying out their intended activity. So well molded are these proteins for the attachment of the hemes that it is indeed possible to remove the hemes by treatment with acid-acetone (43), recombine the apoprotein with heme, and, in the case of hemoglobin (46) and peroxidase (60), reconstitute the molecules and enable them to regain their activity. Apparently, the binding site for the heme in hemoglobin is so constructed that the porphyrin fits into it even when the iron atom has been taken out of the heme. This is demonstrated by the fact that one mole of globin will react with four moles of protoporphyrin (47), just as it will react with four moles of hemin.

When the heme is removed from catalase, and the resulting apocatalase recombined with hemin, it is observed again that four moles of the hemin react with one mole of protein; the activity, however, is not regained. The rotatory dispersion of intact catalase (42) gives a Cotton effect in the region of its iron absorption, whereas the "reconstituted catalase" does not (Fig. 16); these data indicate that the reconstituted catalase molecule is denatured. Yet, the stoichiometry of the reaction reveals that the site of interaction of protein with heme has remained intact, indicating that this site is so stable that even extensive damage to the remainder of the molecule leaves it relatively unharmed.

In spite of the great stability of the hemoproteins, they are eventually destroyed; the heme of hemoglobin, for example, is converted into biliverdin and bilirubin (35), which are tetrapyrroles derived from protoporphyrin, with one of the connecting carbon atoms removed so as to break the porphyrin ring. The cleavage of the porphyrin is accomplished by oxidation in the reticuloendothelial system, the iron is released and presumably finds its way into siderophilin or ferritin to be used again in the synthesis of new molecules of hemoglobin.

REFERENCES

1. BALL, E. G. *Biochem. Ztschr.* 295: 262, 1938.
2. BENNETT, B., J. R. CANNON, A. W. JOHNSON, I. SUTHERLAND, A. R. TODD AND E. L. SMITH. *Nature, London* 176: 328, 1955.
3. BERGEL, F. AND R. C. BRAY. *Biochem. Soc. Symposia* Cambridge, Engl. No. 15: 64, 1958.
4. CHANCE, B. In: *The Enzymes* (1st ed.), Vol. I, edited by J. B. Sumner and K. Myrbäck. New York: Acad. Press, 1951, p. 817.
5. CHRISTENSEN, H. N. AND T. R. RIGGS. *J. Biol. Chem.* 220: 265, 1956.
6. EDLBACHER, S. AND P. BONEM. *Hoppe-Seyler's Ztschr. physiol. Chem.* 145: 69, 1925.
7. EHRENBERG, A. AND C. B. LAURELL. *Acta Chem. Scand.* 9: 68, 1955.
8. EICHHORN, G. L. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 19.
9. EICHHORN, G. L. AND J. C. BAILAR, JR. *J. Am. Chem. Soc.* 75: 2905, 1953.
10. EICHHORN, G. L. AND J. W. DAWES. *J. Am. Chem. Soc.* 76: 5663, 1954.
11. EICHHORN, G. L. AND N. D. MARCHAND. *J. Am. Chem. Soc.* 78: 2688, 1956.
12. EICHHORN, G. L. AND I. M. TRACHTENBERG. *J. Am. Chem. Soc.* 76: 5183, 1954.
13. FISCHER, H. AND H. ORTH. *Die Chemie des Pyrrols.* Vol. 2. Leipzig, Akad. Verlags., 1937, p. 396.
14. GABRIO, B. W., A. SHODEN AND C. A. FINCH. *J. Biol. Chem.* 204: 815, 1953.
15. GARIBALDI, J. A. AND J. B. NEILANDS. *Nature, London* 177: 526, 1956.
16. GEORGE, P. *Conference on Chelation Phenomena.* New York Academy of Sciences, December 1959.
17. GLAUSER, S. Thesis, University of Pennsylvania, Philadelphia, 1959, and private communication.
18. GUBLER, C. J. *Science* 123: 87, 1956.
19. HAHN, P. F., S. GRANICK, W. F. BALE AND L. MICHAELIS. *J. Biol. Chem.* 150: 407, 1943.
20. HEDBOM, A. *Biochem. J.* 74: 307, 1960.
21. HILL, R. AND D. KEILIN. *Proc. Roy. Soc., London*, ser. B. 107: 286, 1930.
22. HOCH, F. L., R. J. P. WILLIAMS AND B. L. VALLEE. *J. Biol. Chem.* 232: 453, 1958.
23. HODGKIN, D. C., J. PICKWORTH, J. H. ROBERTSON, K. N. TRUEBLOOD, R. J. PROSEN AND J. G. WHITE. *Nature, London* 176: 325, 1955.
24. HOLMBERG, C. G. AND C. B. LAURELL. *Acta Chem. Scand.* 1: 944, 1947.
25. IRVING, H. AND R. J. P. WILLIAMS. *Nature, London* 162: 746, 1948.
26. JAEGER, F. M. AND H. B. BLUMENDAL. *Z. anorg. allgem. Chem.* 175: 161, 1928.
27. KENDREW, J. C., G. BODO, H. M. DINTZIS, R. G. PARRISH, H. W. WYCKOFF AND D. C. PHILLIPS. *Nature, London* 181: 662, 1958.
28. KENDREW, J. C., R. E. DICKERSON, B. E. STRANDBERG, R. G. HART, D. R. DAVIES, D. C. PHILLIPS AND V. C. SHORE. *Nature, London* 185: 422, 1960.
29. KROLL, H. *J. Am. Chem. Soc.* 74: 2036, 1952.
30. KUBOWITZ, F. *Biochem. Ztschr.* 292: 221, 1937.
31. KUBOWITZ, F. *Biochem. Ztschr.* 299: 32, 1939.
32. LAUFBERGER, M. *Bull. soc. chim. biol.* 19: 1575, 1937.
33. LAURELL, C. B. In: *The Plasma Proteins*, Vol. I, edited by F. W. Putnam. New York: Acad. Press, 1960, p. 349.
34. LAURELL, C. B. AND B. INGELMAN. *Acta Chem. Scand.* 1: 770, 1947.
35. LEMBERG, R. AND J. W. LEGGE *Hematin Compounds and Bile Pigments.* New York: Interscience, 1949, p. 503.
36. LUKTON, A. AND H. S. OLCOTT. *Biochim. et biophys. acta* 32: 267, 1959.
37. MARGOLIASH, E. *Nature, London* 175: 293, 1955.
38. MICHAELIS, L. *Advances Protein Chem.* 3: 53, 1947.
39. MICHAELIS, L., W. D. CORYELL AND S. GRANICK. *J. Biol. Chem.* 148: 463, 1943.
40. NICHOLAS, D. J. D. AND A. NASON. *J. Biol. Chem.* 207: 353, 1954.

41. NICHOLAS, D. J. D. AND A. NASON. *J. Biol. Chem.* 211: 183, 1954.
42. OSHBAHR, A. J. Thesis, Georgetown University, Washington, D. C., 1960.
43. PAUL, K. G. In: *The Enzymes* (2nd ed.), Vol. 3, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 273.
44. PAULING, L. AND C. D. CORYELL. *Proc. Nat. Acad. Sc., U. S.* 22: 210, 1936.
45. PERUTZ, M. F., M. G. ROSSMANN, A. F. CULLIS, H. MUIRHEAD, G. WILL AND A. C. T. NORTH. *Nature, London* 185: 416, 1960.
46. ROSSI-FANELLI, A. AND E. ANTONINI. *Arch. Biochem. Biophys.* 80: 299, 1959.
47. ROSSI-FANELLI, A., E. ANTONINI AND A. CAPUTO. *Biochim. et biophys. acta* 35: 93, 1959.
48. SCHULTZE, H. E. *Deutsche med. Wchnschr.* 83: 1742, 1958.
49. SINGER, T. P. AND V. MASSEY. *Rec. Chem. Progr.* 18: 201, 1957.
50. SNELL, E. E. *J. Am. Chem. Soc.* 67: 194, 1945.
51. SNELL, E. E. *J. Am. Chem. Soc.* 74: 979, 1952.
52. SPECK, J. F. *J. Biol. Chem.* 178: 315, 1949.
53. STEINBERGER, R. AND F. H. WESTHEIMER. *J. Am. Chem. Soc.* 71: 4158, 1949.
54. STEINBERGER, R. AND F. H. WESTHEIMER. *J. Am. Chem. Soc.* 73: 429, 1951.
55. TAKERU, I. AND J. B. NEILANDS. *J. Am. Chem. Soc.* 80: 4645, 1958.
56. THEORELL, H. *Biochem. Ztschr.* 298: 242, 1938.
57. THEORELL, H. *Biochem. Ztschr.* 301: 201, 1939.
58. THEORELL, H. AND Å. ÅKESSON. *J. Am. Chem. Soc.* 63: 1818, 1941.
59. THEORELL, H. AND Å. ÅKESSON. *J. Am. Chem. Soc.* 63: 1804, 1941.
60. THEORELL, H., S. BERGSTROM AND S. AKESSON. *Arkiv. Kemi., Mineral. Geol.* 18A: No. 12, 1944.
61. TUPPY, H. AND G. BODO. *Monatsh. Chem.* 85: 807, 1954.
62. TUPPY, H. AND G. BODO. *Monatsh. Chem.* 85: 1024, 1954.
63. TUPPY, H. AND S. PALËUS. *Acta Chem. Scand.* 9: 353, 1955.
64. ULMO, D. O. AND B. L. VALLEE. *Fed. Proc.* 19: 27, 1960.
65. VIKBLADH, I. *Scandinav. J. Clin. & Lab. Invest.*, Supp. 2: 3, 1951.
66. WARNER, R. C. AND I. WEBER. *J. Am. Chem. Soc.* 75: 5094, 1953.
67. WESTHEIMER, F. H. In: *The Enzymes* (2nd ed.), Vol. I, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 273.
68. WILLIAMS, R. J. P. In: *The Enzymes* (2nd ed.), Vol. I, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 412.

DISCUSSION

Stereochemical aspects of alkaline earth chelation by polyamineacetic acid[1]

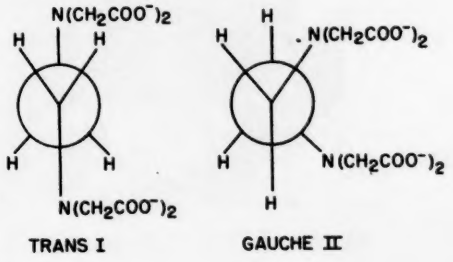
HARRY KROLL AND MARIA GORDON

Division of Neoplastic Diseases, Montefiore Hospital, New York City

THE CONFORMATIONAL ANALYSIS of chelate rings is of considerable value in evaluating the contribution of the structure of organic chelating agents to metal chelate stabilities and metal-binding specificities (6). This aspect of metal chelate chemistry was studied by determining the effect of modifications in the spatial arrangement of the metal-binding atoms of a group of polyamineacetic acids on the chelation of alkaline earth cations in water.

The ethylenediaminetetraacetate anion must have a staggered or *trans* configuration I with respect to the iminodiacetate groups. The chelation of a metal ion requires that a rotation of the ethane carbon atoms takes place so that the metal chelate assumes the *gauche* structure II. A true *cis* or eclipsed configuration of the iminodiacetate group is not possible since this would require an overlapping of the hydrogen atoms on the ethane carbons (2). If the *gauche* configuration represents the structure of an EDTA metal chelate, then the chelate ring containing the 1,2-diamine metal complex is not planar. The deviations from planarity should impose a strain on the cyclic structure which should be evident in the stability of the metal chelate. It is important to determine whether this stereochemical influence on metal chelation can be utilized to produce metal-binding specificities, particularly between the biologically interesting cations, magnesium, calcium, and strontium.

The carboxymethylated derivatives of carbocylic 1,2-



[1] This investigation was supported by the U. S. Atomic Energy Commission, Contract AT (30-1)2094.

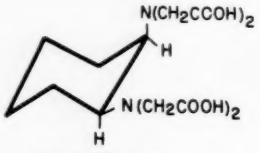
TABLE 1. *Stability Constants (Log K_1) of Cis 1,2-Diaminocyclohexanetetraacetic Acid (Cis CDTA), Trans 1,2-Diaminocyclohexanetetraacetic Acid (Trans CDTA), and Trans 1,2-Diaminocyclopentanetetraacetic Acid (Trans CPDTA)**

	Cis CDTA	*Trans* CDTA	*Trans* CPDTA
pk_1	2.44	2.43†	1.75
pk_2	3.50	3.52†	2.63
pk_3	5.21	6.12†	7.44
pk_4	10.70	11.70†	10.26
		Log K_1	
Mg	8.38	10.32†	9.05
Ca	9.45	12.50†	11.08
Sr	7.33	10.69	9.45

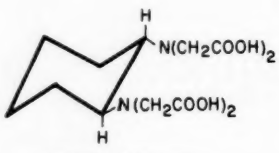
* Measurements made at 20° C, I = 0.1.

† Schwarzenbach, G. and H. Ackermann. *Helv. Chim. Acta* 32: 1682, 1949.

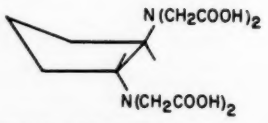
diamines represent a group of compounds having fixed stereochemical configurations. These compounds have a high affinity for the alkaline earths and differ from one another by the separation of the diamine nitrogens and the position of these atoms relative to the carbocyclic ring. A comparison of the stability constants of the alkaline earth chelates offers a convenient method of analyzing these stereochemical contributions to the chelation reaction.



CIS CDTA III



TRANS CDTA IV



TRANS CPDTA V

The stability constants of the alkaline earth chelates of *cis* and *trans* 1,2-diaminocyclohexanetetraacetic acid (*cis* CDTA, III, and *trans* CDTA, IV) and *trans* 1,2-diaminocyclopentanetetraacetic acid (*trans* CPDTA, V) are listed in Table 1. The order of these stabilities is *trans* CDTA > *trans* CPDTA > *cis* CDTA. This stability sequence is not in the same order as the distances separating the ligand nitrogen atoms. The separation of the diamine nitrogens in this group of compounds is: *cis* CDTA ≦ *trans* CDTA < *trans* CPDTA. The reduced stabilities of the alkaline earth chelates of *cis* CDTA are attributed to the stereochemical interference with the chelation reaction. Models of *trans* CDTA (Fig. 1*A*) and *trans* CPDTA indicate that they form their metal chelate rings in a plane adjacent to the carbocyclic rings without interference from these structures. In contrast to the *trans* isomer, a model of *cis* CDTA (Fig. 1B) shows that the axial hydrogens of the cyclohexane ring interfere with the free rotation of the carboxymethyl groups, and the plane of the chelate ring is at an oblique angle to the plane of the carbocyclic structure. Further, the equatorial and axial positions occupied by the nitrogens in *cis* CDTA increase the nonplanarity of the 1,2-diamine metal chelate ring, as compared to the diequatorially orientated ring in *trans* CDTA.

It is interesting to note that the alkaline earth chelates of *trans* CPDTA are more stable than the corresponding compounds of ethylenediaminetetraacetic acid even though the distance separating the diamine nitrogens in the *gauche* configuration of the EDTA chelate is less than the corresponding distance in the *trans* CPDTA. The decreased stabilities of the EDTA chelates are attributed to the energy required to convert the *trans* form to the *gauche*. In the *trans* CPDTA, the nitrogens are spatially fixed by the cyclopentane ring eliminating the rotational energy requirement. The conclusions from these studies on the carbocyclic 1,2-diaminetetraacetic acids are that the stabilities of the alkaline earth chelates can be influenced by the spatial arrangement of the ligand nitrogens, but no outstanding preferences for

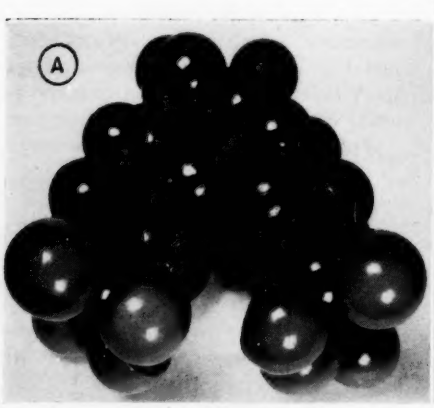


FIG. 1*A*. *Trans* CDTA.

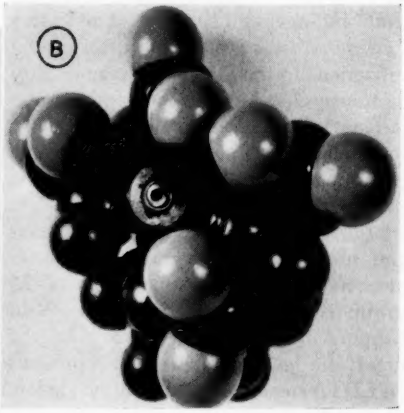
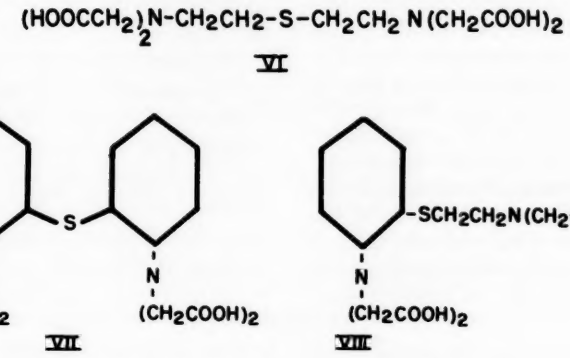


FIG. 1*B*. *Cis* CDTA.



any of the alkaline earth cations are observed for these stereochemical modifications.

The compound, bis-[N,N′-di(carboxymethyl)2-aminoethyl] sulfide, VI, forms a strontium chelate which approaches the stability of the corresponding calcium compound (5). The thioether tetraacetic acid would be of interest in removing strontium-90 from physiological systems, but it is ineffective for this application because of the instability of the calcium and strontium chelates at pH 7.5 and below (Log K $\sim$ 4). The enhanced stabilities of the alkaline earth chelates of *trans* CDTA prompted an investigation of the effect of introducing the cyclohexane structure in VI. The compounds bis-[N,N′-di(carboxymethyl)2-aminocyclohexyl] sulfide (3), VII, and N-di(carboxymethyl)2-aminoethyl-N′-di(carboxymethyl)2-aminocyclohexyl sulfide (4), VIII, were synthesized. A slight increase in the stabilities of the calcium and strontium chelates was observed, but the effect was too small to exert any physiological response. The increase in chelate stability appeared to be greater for calcium than strontium. It was concluded that stereochemical modifications of VI had only a small effect on increasing alkaline earth chelate stabilities. The limiting factor in these molecules appears to be the metal-binding affinity of the sulfur atoms for the alkaline earth cations.

TABLE 2. *Stability Constants (Log K_1) of Alkaline Earth Chelates of Bis[di(carboxymethyl)aminoethyl] Ether (BAETA) and Bis[di(carboxymethyl)2-aminopropyl] Ether (1-Methyl BAETA)**

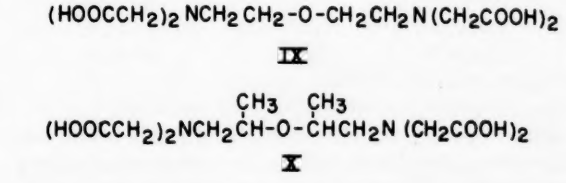
	BAETA†	Methyl BAETA
pk_1	1.75	2.69
pk_2	2.76	3.04
pk_3	8.84	8.72
pk_4	9.47	9.45
	Log K_1	
Mg	8.31	7.92
Ca	10.05	8.06
Sr	9.34	7.17
Ba	8.15	5.88

* Measurements made at 20° C, I = 0.1.

† Schwarzenbach, G. In: *Stability Constants. Pt. 1: Organic Ligands*, edited by J. Bjerrum, G. Schwarzenbach and L. G. Sillén. London: The Chemical Society, 1957, p. 84.

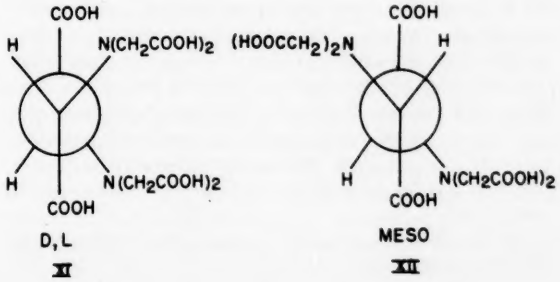
A recent report by West (1) has disclosed that one of the isomeric 2,3-diaminobutanetetraacetic acids forms metal chelates having approximately the same order of stability as *trans* CDTA, whereas the other isomer is much weaker. A conformational study of models of these two molecules indicates that the racemic form must form the more stable metal chelates. This conclusion is based on the assumption that the 2,3-diaminobutanetetraacetic acids will assume a conformation in which the methyl groups are as far apart as possible in a *trans* planar structure.

An attempt was made to evaluate this effect on the alkaline earth chelation of a methyl substituted N,N′-bis-[di(carboxymethyl)2-aminoethyl] ether, IX. A comparison of the stability constants of the alkaline earth chelates of N,N′-bis-[di(carboxymethyl)2-aminopropyl] ether, X, and IX are shown in Table 2. The introduction of



methyl substituents produced a marked drop in metal chelate stability. The interpretation of these data must await the elucidation of the configurations of the asymmetric carbon atoms containing the methyl substituents.

Another example of the stereochemical influence on metal chelation is illustrated by N,N,N′N′-bis-di(car-



boxymethyl)*meso*-1,2-diaminosuccinic acid, XII. This compound has a low affinity for the alkaline earth cations. The conformational structure assigned to XII places the carboxylate and iminodiacetate groups in *trans* positions. The formation of the metal chelate of XI requires that the negatively charged carboxylate groups be adjacent to one another. This unfavorable conformation is evidenced by a log K value for the calcium chelate of approximately three. A clean cut synthesis of the racemic isomer XI has not yet been achieved, but it is predicted that this compound will exhibit metal chelate stabilities for the alkaline earths of the same order as *trans* CDTA.

The conclusions from these studies are that the absolute magnitude of the stabilities of the alkaline earth chelates can be varied considerably by stereochemical modifications of the organic ligand, but these changes are of approximately the same order for each of the alkaline earth cations. No preferential metal-binding has been observed as a result of the structural modifications discussed in this report.

REFERENCES

1. Belcher, R., W. Hoyle and T. S. West. *Talanta* 3: 201, 1959.
2. Dauben, W. G. and K. S. Pitzer. In: *Steric Effect in Organic Chemistry*. New York: Wiley, 1956, p. 6.
3. Kroll, H. *Annual Report, U. S. Atomic Energy Commission, Contract AT(30-1)2094*. AECU-4322, 1959.
4. Kroll, H. *Annual Report, U. S. Atomic Energy Commission, Contract AT(30-1)2094*. TID-6925, 1960.
5. Schwarzenbach, G. In *Stability Constants. Pt. 1. Organic Ligands*, edited by J. Bjerrum, G. Schwarzenbach and L. G. Sillén. London: The Chemical Society, 1957, p. 85.
6. Wilkins, R. G. and M. J. G. Williams. In: *Modern Coordination Chemistry*. New York: Interscience, 1960, p. 178.

DISCUSSION

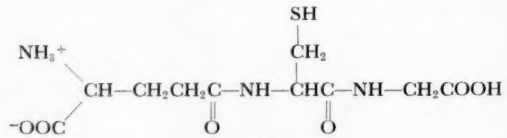
Metal ion binding to peptides and proteins[1]

R. BRUCE MARTIN

Department of Chemistry, University of Virginia, Charlottesville, Virginia

BINDING TO GLUTATHIONE

The study of metal ion binding to peptides is valuable for two reasons: for the information obtained on binding to peptides themselves and for the insight given as to what may be expected when metal ions are bound to proteins. The direct study of the binding of an individual metal ion to a particular group in a protein is usually not possible. Therefore, model compounds have often been utilized in order to assess the metal-binding capacity. For example, the strength of the metal ion binding to the imidazole molecule has been used successfully as a model for the estimation of the binding to the histidyl residue of proteins. The sulfhydryl bearing cysteinyl residue lacks a model molecule because all small molecules bearing a sulfhydryl group either precipitate with many metal ions or have an additional chelating group, such as the amino group, from which it is not possible to ascertain unequivocally the contribution of the sulfhydryl group. For these reasons, the sulfhydryl group of cysteine is the most difficult side chain group of proteins to assess quantitatively for its metal ion binding capacity. A quantitative study of the metal-binding capacity of the tripeptide glutathione is doubly rewarding: the metal-binding of the naturally occurring compound itself is of interest and the middle cysteinyl residue bound on both sides by peptide bonds makes glutathione a nearly perfect model for the sulfhydryl group of proteins.



Glutathione

In order to make a quantitative study of the metal ion binding to glutathione it is necessary to separate the competing effects of the γ-glutamyl locus and the sulfhydryl group for both hydrogen and metal ions. In the course of the study the selectivity of these two areas of the glutathione molecule for cations has been evaluated. Thus the selectivity of metal ions for two different groups

[1] This research was supported by a grant from the National Science Foundation (G9796).

has been determined on the same molecule. This was accomplished by a study of the titration curves of S-methylglutathione in the presence and absence of divalent metal ions. The contribution of the γ-glutamyl locus was determined on the assumption that the ether sulfur is equivalent to an unionized sulfhydryl group. Quantitative comparison of the titration curves with those of glutathione with and without metal ions yield, by standard methods of analysis, the results of Table 1 (6, 7).

In the second column of Table 1, log k_N is the formation constant for cation binding at the γ-glutamyl locus with the sulfhydryl group still protonated. In the third column, log k_S is the formation constant at the sulfhydryl group with the ammonium group in the cationic form. These are the relevant constants for the predominant species in solution at physiological pH values. Finally, in the fourth column, an estimate is made of the percentage of the cation at the sulfhydryl group, assuming a deficit of the cation. If sufficient cation were available both sites would be completely occupied. Unfortunately, the values of log k_S for the last three cations depend upon the subtraction of two large numbers and are uncertain. The values quoted for "% cation at S" in the last column are, therefore, only approximations for Ni^{++}, Co^{++} and Mn^{++}.

The values of log k_S in Table 1 are less than those usually assumed. The greater value for zinc as compared with nickel ion is a reversal of the usual order (observed in log k_N) for most ligands of interest to proteins. This alternation is readily apparent from the titration curves. For glutathione the zinc curve is at lower pH values than the nickel curve whereas the reverse is true for S-methylglutathione (7).

It is interesting to note that any chelation occurring in glutathione by the metal ion between the sulfhydryl group and the elements of a peptide bond presumably also would occur in proteins. Thus glutathione is a superb model for measuring the combining capacity of metal ions to the unmasked sulfhydryl groups of proteins.

Two tests are suggested by the above study for the participation of sulfhydryl groups in enzyme reactions. The effect of zinc should be greater than that of nickel ion and the dissociation constant of the enzyme-zinc ion complex should be calculated and compared with the value of 10^{-5}. For several inhibition and activation studies on enzymes both of these criteria are satisfied and, therefore, the sulfhydryl group presumably is involved critically in some way. If the inhibition by the metal ion is competitive, the enzyme-metal ion dissociation constant, K_M, may be evaluated by standard methods. For the metal ion concentration (M) that reduces the velocity by one-half, $K_M = K_s(M)/(S)+K_s$, where (S) is the substrate concentration and K_s the Michaelis-Menten constant. Thus, in general, $K_M \neq (M)$ for one-half velocity unless $K_s \gg (S)$.

The significance of glutathione in biological systems is still not certain. Glutathione with two metal-binding sites may function as a protective agent for enzymes against metal ions. Conversely, glutathione may bind metal ion enzyme activators and explain inverse relationships between activity and glutathione concentration.

TABLE 1. *Logarithm of Formation Constants for Cations with S-Methylglutathione and Glutathione at 25° and 0.16 μ**

Cation	Log k_N	Log k_S	% Cation at S
H^+	9.20	8.92	35
Zn^{++}	4.65	5.00	80
Ni^{++}	5.25	4.0	10
Co^{++}	4.30	3.7	30
Mn^{++}	2.85	1.9	15

* Reference 7.

INTERACTIONS AT PEPTIDE BONDS

The most obvious feature of proteins is the repetition of amide bonds. In strongly alkaline solutions these bonds react with cupric ions to give a violet color and with nickel ions to yield a yellow color, often called the biuret reaction. These reactions involve the ionization of amide hydrogens. It has been known for some time that divalent copper ion, upon combination with the terminal amino group of simple peptides, induces by chelation the ionization of the nitrogen bound hydrogen of the amide bond at less than physiological pH values (see Reference 5 for references). More recently, a similar interaction has been unequivocally demonstrated for divalent nickel ion (5). Comparison of the results obtained with either metal ion with glycylglycine and with glycylsarcosine indicate that it is the amide hydrogen and not an ionization of a proton from the hydration sphere of the metal ion that is responsible for the titration of an additional mole of hydrogen ion. In the case of glycinamide and tri- and higher peptides the biuret colors often are obtained at about physiological or slightly greater pH values.

In mixtures of divalent nickel ion and glycinamide, triglycine or tetraglycine the solution turns from blue to yellow on the ionization of the amide hydrogens from the metal ion-peptide complexes. For the latter two peptides the ionization of the first amide hydrogen promotes the ionization of the remainder so that the ionizations occur in a less than statistical fashion. Thus the second amide hydrogen ionizes more easily than the first despite the fact that the complex is more negative after the first ionization. In the case of cupric ion and the same peptide, the ionizations do not occur in a less than statistical fashion. The result is that the titration curves of tetraglycine with copper and nickel ions cross, the nickel complex becoming the more stable after the peptide hydrogens have ionized. For the tetraglycine complex of nickel ion, a definite time factor is evident for equilibrium to be attained in either potentiometric or spectrophotometric experiments, involving the amide hydrogen ionizations. These facts imply that a profound molecular rearrangement takes place on the ionization of the amide hydrogens as evidenced by the markedly less than statistical ionizations, increasing yellow color and the relatively long time for attainment of equilibrium (5).

In the stereochemistry of divalent nickel ion two main types of complexes are usually considered; blue, octahedral, paramagnetic complexes and yellow, square planar, diamagnetic complexes. The formation of the yellow color on the ionization of the amide hydrogens indicates that the peptide wraps around the nickel ion in a plane. In the case of tetraglycine, a nitrogen atom is at each corner of the plane. The less than statistical ionizations mean that, as the titration proceeds past the addition of the first equivalent of base per mole of metal ion, part of the complexes are ionized completely with the others simply chelated rather than the condition where all of the complexes are partly ionized.

In order to further elucidate the structure, the magnetic susceptibility of the yellow nickel-triglycine complex was measured. The solid complex was prepared by adding three moles of sodium hydroxide to a solution containing equimolar amounts of nickel chloride and triglycine. The solvent was evaporated and the weight of the solid remaining was accounted for by the triglycine, nickel, sodium, and chloride ions added. Therefore no water of hydration or hydroxide ions remained. A slight excess of triglycine and of base was added to make sure that a slight dissociation would not contribute to the paramagnetic susceptibility. The magnetic susceptibility of the solid was measured by Drs. Rolf B. Johannesen and George Candela. They determined the susceptibility by the absolute Faraday method (11) at two temperatures and corrected for diamagnetic contributions. The corrected molar susceptibilities are 861×10^{-6} at 26° C and 4493×10^{-6} at −195.5° C. By the simple Curie Law 1.44 and 1.67 Bohr magnetons are obtained at the two temperatures, respectively. The Curie-Weiss Law constants are 1.37 Bohr magnetons with the Weiss constant, $\Delta = -25°$.

The expected spin of magnetic moment of paramagnetic divalent nickel ion complexes with two unpaired electrons is 2.83 Bohr magnetons. Therefore, the observed value of about 1.4 Bohr magnetons is too large for a diamagnetic complex and too small for a fully paramagnetic complex. The observed moment is a little lower than the 1.73 Bohr magnetons expected for one unpaired electron in an unlikely tervalent nickel complex. This intermediate result as well as the negative Weiss constant suggest that a detailed study of the temperature dependence of the magnetic susceptibility from liquid helium temperatures might be informative. A similar study on the nickel-tetraglycine complex should also yield interesting and probably different results because of the stronger crystal field due to the ionization of three amide hydrogens. The yellow nickel complex of 2,3-dimethyl-2,3-diaminobutane (see Table 2) is diamagnetic (2). The explanation for the intermediate value of the magnetic susceptibility for the triglycine complex may be a diamagnetic-paramagnetic equilibrium of square planar complexes (1, 9, 10). However, the ultimate determination of the configuration may depend on an X-ray diffraction study. Nuclear magnetic resonance studies might also be of interest. Many future experiments are suggested on the stereochemistry of the nickel ion complexes of peptides by the results.

TABLE 2. *Wavelength Maxima and Molar Extinction Coefficients of Divalent Nickel Ion Complexes*

	λ_{max}	ϵ_{max}
Glycinamide*	438	61
Triglycine*	430	240
Tetraglycine*	412	215
Glycylhistidine†	450	110
Histidylhistine†	450	110
BSA (asp-thr-)	425	130
HSA (asp-ala-his-‡)	418	130
Biuret	467	57
2,3-Dimethyl-2,3-diaminobutane§	434	64

* Reference 5. † Reference 8. ‡ For N terminal sequence, Ikenaka, T. *J. Am. Chem. Soc.* 82: 3180, 1960. § Reference 2.

Are the above effects of nickel-peptide complexes observed in solutions of proteins and nickel ion? It has been reported (3) that at pH 9.2 the initial reaction of one mole of cupric ion with one mole of bovine serum albumin (BSA) is at some site other than the sulfhydryl group. It was postulated that this interaction site may be the terminal aspartic acid group. Nickel ion inhibits the interaction of cupric ion at low nickel to cupric ion ratios, whereas zinc ion has practically no inhibitory effect (3).

The high degree of inhibition for the low ratio of nickel to cupric ions and the lack of inhibition by zinc ion suggest that perhaps the strong nickel ion interactions that take place in peptides mentioned above occur also in BSA. Recrystallized BSA (Mann Research Labs) was mixed with nickel ion in varying ratios at about pH 10. A spectrophotometric analysis of the yellow complex formed indicated a 1:1 complex of BSA and nickel ion. A study of equimolar mixtures of BSA and nickel ion showed that a pH of about 9.5 was required for the full development of the yellow color. Since this is much less alkaline than that required for the usual biuret type complexes of nickel ion, a special type of interaction may be inferred.

In accord with the results on the peptide complexes of nickel ion and the 1:1 interaction of BSA and nickel ion, association at the N terminus followed by chelation and ionization of amide hydrogens seems very likely. The inhibitory effects of nickel on the cupric ion association with BSA previously reported (3) thus are due to the same strong interactions observed in peptides. These observations and conclusions support the assumption that the first mole of cupric ion reacts at the N terminus of BSA.

In Table 2 are listed the wavelength maxima for various yellow complexes of nickel ion with molar extinction coefficients of the order of 100. The results for BSA and HSA (human serum albumin) are the least accurate because the augmentation of the residual yellow color due to the formation of the nickel ion complex was less than 40% for both albumins. The N terminal sequence for the albumins is also listed. Only

the value for the biuret was recorded in a strongly basic (1N) solution.

The powerful interaction of divalent nickel ions with amide hydrogens in more or less neutral solutions has been observed in several peptides (4, 5, 8) and in two proteins, BSA and HSA. The ionization of amide hydrogens from the nickel complex is not always accompanied by the appearance of a yellow color (4, 5, 8). There would seem to be no reason why the association of either divalent copper or nickel ions at the N terminus of a protein, followed by chelation and ionization at the neighboring peptide bonds, should not be a general phenomena. The metal ion in such a complex probably would exchange very slowly and the non-exchangeability observed in some copper proteins might be explained at least partly in this way. The interaction could not occur in proteins with an acetylated N terminal residue (TMV protein) or in proteins with proline in the second position (5). However, a histidyl residue in the second (β chain of normal, adult, human hemoglobin) or third (HSA) position might render the interaction more likely (8). A comparison of the binding of cupric and nickel ions to anserine and carnosine indicates that the two ions may react differently with histidyl residues (4). It is surprising that the metal ion induced amide hydrogen ionization is not biologically significant in some way or other. However, for the moment, the interaction remains an interesting reaction in search of a function.

BINDING TO CYTOSINE AND CYTIDINE

The ambitious topic indicated in the title will be briefly expanded to mention the cupric ion binding properties of the nucleic acid base cytosine and the corresponding nucleoside cytidine. Metal ion binding to nucleic acids is thought usually to be a predominantly electrostatic interaction between negatively charged phosphate residues and metal ions such as magnesium. The nitrogen atoms of the bases would be expected to bind metal ions such as cupric much more strongly than metal ions such as magnesium. The study of cupric ion binding with cytosine and cytidine (Calbiochem) was undertaken in order to ascertain the strength of the association of a transition metal ion with a water-soluble, non-chelating nucleic acid base and its corresponding nucleoside. At 25° and 0.16 μ the pKa values are 4.69 and 4.21 for cytosine and cytidine, respectively. The logarithms of the first association constants as determined by standard potentiometric methods are 2.0 and 1.4 for the base and sugar, respectively. The results are not precise due to the expected, relatively weak interaction. There was no tendency for a second mole of cytidine to associate with the equimolar complex. The interaction of zinc ion was very weak with both ligands.

I am grateful to Dr. Rolf B. Johannesen and Dr. George Candela of the National Bureau of Standards for obtaining the magnetic susceptibility measurements on the nickel-triglycine complex.

REFERENCES

1. Ballhausen, C. J. and A. D. Liehr. *J. Am. Chem. Soc.* 81: 538, 1959.
2. Basolo, F., Y. T. Chen and R. K. Murman. *J. Am. Chem. Soc.* 76: 956, 1954.
3. Kolthoff, I. M. and B. R. Willeford, Jr. *J. Am. Chem. Soc.* 80: 5673, 1958.
4. Martin, R. B. *J. Am. Chem. Soc.* in press.
5. Martin, R. B., M. Chamberlin and J. T. Edsall. *J. Am. Chem. Soc.* 82: 495, 1960.
6. Martin, R. B. and J. T. Edsall. *Bull. soc. chim. biol.* 40: 1763, 1958.
7. Martin, R. B. and J. T. Edsall. *J. Am. Chem. Soc.* 81: 4044, 1959.
8. Martin, R. B. and J. T. Edsall. *J. Am. Chem. Soc.* 82: 1107, 1960.
9. Maki, G. *J. Chem. Phys.* 29: 1129, 1958.
10. Sacconi, L., R. Cini, M. Ciampolini and F. Maggio. *J. Am. Chem. Soc.* 82: 3487, 1960.
11. Thorpe, A. and F. E. Senftle. *Rev. Sci. Instr.* 30: 1006, 1959.

GENERAL DISCUSSION

Dr. Saltman: For the past several years, we have been concerned with the mechanisms of iron transport in mammalian systems. We came across an interesting phenomenon which I think highlights and emphasizes Dr. Eichhorn's statement that chelation may play an important role in transport. Initially, we measured the uptake of iron by rat liver slices and found that this system was independent of metabolic energy and, yet, could lead to the establishment of apparent concentration gradients of the metal within the cell (Saltman, P., R. D. Fiskin and S. B. Bellinger. *J. Biol. Chem.* 220: 741, 1956; Saltman, P., R. D. Fiskin, S. B. Bellinger and T. Alex. *J. Biol. Chem.* 220: 751, 1956; Saltman, P., H. L. Frisch, R. D. Fiskin and T. Alex. *J. Biol. Chem.* 221: 777, 1956). In these experiments the iron was presented as ferric ammonium citrate. The presence of any other chelate for iron in reasonable concentration competed with the slice for the metal and inhibited uptake. Recently, we have carried out even more definitive experiments to demonstrate the role of chelation by measuring the flux of Fe^{59} from transferrin into the liver slice (Charley, P., M. Rosenstein, E. Shore and P. Saltman. *Arch. Biochem. Biophys.* 88: 222, 1960). We find a direct dependence of the initial rate of uptake on the concentration of the chelate. In these experiments we used both EDTA and citrate.

Furthermore, there needs to be some clarification of the

participation of ferritin in iron transport. There is voluminous literature relating to the absolute necessity for ferritin to facilitate the transport of iron through the mucosal cell and into the circulating serum. In recent experiments (Charley, P. and P. Saltman. *Fed. Proc.* 19: 248, 1960) we have devised a rabbit preparation in which we were able to monitor instantaneously the movement of the Fe^{59} from the lumen of a washed gut loop into the circulating blood. There is a complete absence of any lag period. The ferritin does not seem to participate in the process. But we found that the chemical nature of the chelate used to introduce the Fe^{54} is the most significant aspect of the transport problem. If the iron is in the form of a low molecular weight, uncharged complex, it moves very rapidly across the mucosal wall. If it is charged or in a form which readily precipitates at the alkaline pH of the gut, it is no longer moved. These experiments speak very strongly and clearly for the participation of chelate molecules in the transport process of many heavy metals.

DR. CALVIN: Dr. Eichhorn, have you correlated rotatory dispersion curves with activity studies?

DR. EICHHORN: In a previous paper we had shown that the rotatory dispersion of catalase at pH 11 is also simple rather than a Cotton effect curve. At pH 11, the optical rotation values actually are of the same order of magnitude as those of the intact molecule. It was with acid catalase that we got rotatory dispersion curves in which the rotation values were much more negative than those of intact catalase. The pH 11 catalase curve, however, looks very much like the curve of the so-called reconstituted catalase. The activity of both the pH 11 catalase and the so-called reconstituted catalase is zero. I have a feeling that it should be possible to get a reconstituted catalase molecule with some activity. One of the difficulties is that, in this titration of apocatalase with hemin, one goes through an isoelectric point and gets a precipitate which then dissolves. If we could use a porphyrin other than protoporphyrin which is more soluble, I have a feeling that we could get some activity. Similar things have been done with hemoglobin. The removal of the heme from the catalase was done by using the acetone-acid splitting technique, which has been modified recently by Rossi-Fanelli and co-workers for removing heme from hemoglobin. Apparently, they were able to get a preparation of globin which is very much purer than any which has hitherto been obtained. They were able to reconstitute hemoglobin with virtually the same physical and chemical properties as the native hemoglobin. We were not able to duplicate this with catalase.

DR. LEON L. WIESEL (New York State University, Downstate Medical Center, Brooklyn, N. Y.): I would like to ask Dr. Eichhorn a question about which I am puzzled. He began by saying that copper was bound too powerfully to both proteins and substrates to be active as a metal factor in enzyme action. You indicated that iron was the active ion. Yet, at least half of the electron transport enzymes are copper-dependent.

DR. EICHHORN: I did not mean to imply that copper is not a very important constituent of many enzymes. What I did mean is that in many nonenzymatic reactions the order of activity is in the order of the Irving-Williams series, according to which copper should be the best activator. This order is not followed by most enzymatic reactions. In fact, many enzymatic reactions are inhibited by copper. On the other hand, many other enzymatic reactions, of course, are activated by copper.

DR. ALBERT: There is the important matter of metal-binding in nucleic acid. Apart from one paper on calcium, magnesium, and manganese (Wiberg, J. S. and W. F. Neuman. *Arch. Biochem. Biophys.* 72: 66, 1957), very little is known about the stability constants for nucleic acids which are very large molecules and difficult to examine. What one can do is to look at individual nucleotides. The pyrimidine nucleoside, cytidine, shouldn't be able to chelate because of its structure and, in fact, it doesn't. On the other hand, the purine nucleosides, guanosine (Albert, A. *Biochem. J.* 54: 646, 1953) and adenosine (Harkins, T. R. and H. Freiser. *J. Am. Chem. Soc.* 80: 1132, 1958) are capable of considerable chelation.

DR. WILLIAMS: I wish to draw Dr. Eichhorn's attention to two papers: *Chem. Revs.* 56: 299, 1956 and *Biol. Revs. Cambridge Phil. Soc.* 28: 381, 1953. In these papers I advance views on acid-base catalysis by metal ions and electron transport in porphyrin complexes which are in close conformity with those advanced by Dr. Eichhorn.

DR. HENRY FREISER (University of Arizona): Dr. Kroll, what are the pK_a values of the *cis* and *trans* CDTA, and of the cyclopentane analog? Might there be sufficient difference in the respective pK_a values to account for the chelate stability differences you have found?

DR. KROLL: The pK_1 and pK_2 values for the *cis* and *trans* diaminocyclohexanetetraacetic acids are practically identical and have been inserted in the final paper. On the other hand, the pK_3 and pK_4 values indicate that the *cis* isomer is a stronger acid than the *trans*. These results can be explained on the basis of stereochemistry of the cyclohexane ring. I don't remember the distance, in angstroms, that separates the nitrogens in the cyclohexane and cyclopentane derivatives, but they are about 30 to 40% greater for the *trans* diaminocyclopentane than for the *cis* and *trans* diaminocyclohexanes. If models are made of the *trans* diaminocyclohexanetetraacetic acid, an almost planar relation exists between the chelate ring and the cyclohexane ring. In the metal chelates of *trans* diaminocyclopentanetetraacetic acid, there is a similar tendency toward planarity between the chelate ring and the cyclopentane ring. This relationship seems to be a requirement for formation of stable chelates in the carbocyclic 1,2-diaminotetraacetic acids.

DR. SCHUBERT: Dr. Kroll is talking about synthesizing an agent for calcium and strontium with high absolute values of the formation constants. The object, however, is to produce chelating agents in which strontium has a higher formation constant than calcium. Empirically and theoretically, this appears to be very difficult. For example, if one plots log K_1(Ca) *vs* Δlog K_1(Ca-Sr), that is, the difference in log K_1 between Ca and Sr, one never finds a higher value for log K_1(Sr) relative to Ca. This relationship holds even though all the organic chelating agents tabulated in the Bjerrum, Schwarzenbach, Sillén tables (*Stability Constants. Pt. I: Organic Ligands*, edited by J. Bjerrum, G. Schwarzenbach and L. G. Sillén. London: The Chemical Society, 1957) are included. For conventional chemical reasons, as the formation constant of Sr relative to Ca gets smaller so, unfortunately, does the *absolute* values of the formation constant of Sr.

DR. KROLL: What Dr. Schubert says is true. We obtained marked changes in chelate stabilities by varying the stereochemistry, but we did not get any great changes in the relative values between magnesium, calcium, and strontium. Our thinking is that to obtain selectivity we must look more closely at the use of selective functional groups rather than at a class of compounds such as the tetraacetic acids.

DR. FREISER: Dr. Martin, the pK_a values you show for glutathione and its S-methyl derivative are very close. How

were you able to sort out the pK_a values for the former and establish that it represents the ionization of the sulfhydryl proton?

DR. MARTIN: Two problems are raised by this question. Determination, from titration data, of the pK_a values for overlapping ionizations may be performed by several methods, the most convenient of which, to my mind, is that described on page 516 of the paper by J. T. Edsall, R. B. Martin and B. R. Hollingworth (*Proc. Natl. Acad. Sci. U. S.* 44: 505, 1958). Assignment of pK values to individual groups with overlapping ionizations is more difficult and requires information in addition to titration data on the compound. Either spectrophotometric data or the synthesis and titration of model compounds such as S-methylglutathione may provide the necessary additional information. Both methods are described in the paper just mentioned and the application of the overlapping sulfhydryl and ammonium ionizations of glutathione is given in Reference 6 of my paper in this symposium.

DR. MARTELL: In connection with Dr. Martin's description of the nickel(II)-peptide complexes, I would like to add that there is some unpublished work by Schwarzenbach and Martell, done about five years ago, in which the nickel complexes of glycylglycine and glycylglycylglycine were investigated. In agreement with Dr. Martin's results, we also found these compounds to be yellow, and that equilibrium was attained slowly when the metal presumably combined with the peptide linkage of the ligand.

Role of metal-binding in enzymic reactions[1]

BO G. MALMSTRÖM

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

AMONG THE MULTITUDE of functions that metal chelate compounds perform in biological systems, their participation in a great number of enzymic reactions assumes a particular importance. The various roles of metal ions in enzyme catalysis have been discussed in a great number of reviews, and a few recent ones, in which references to older literature can be found, are listed in the references (4, 34, 55, 57, 62). At this Symposium, Dr. Eichhorn has already given a survey of those attributes of chelates that are potentially useful in biological processes. I would here like to supplement his discussion by considering, from an enzymologist's point of view, some major problems and experimental approaches in the study of the parts played by metals in enzymic reactions.

TYPES OF COMPLEXES

Metal ions can influence the rate of enzymic reactions in a great variety of ways by interacting with components in the reaction system. A basic problem in studying such influences is, therefore, a determination of which complexes partake in the reaction sequences of the catalytic process. In case of the true metalloenzymes (54), i.e. enzymes which can be isolated with the metal firmly attached to the protein in stoichiometric amounts, the problem, while requiring rigorous analytical work, is in principle simple. With these enzymes, correlations between three parameters, the protein, the metal, and the activity, clearly show that the metal functions in a complex with the protein. Since this approach for demonstrating the functional involvement of metal ions in this group of enzymes will be thoroughly discussed by Dr. Vallee at this Symposium, it will not be considered further here. It should, however, be noted that studies on the function of the metal in metalloenzymes often are hampered by the firmness of the attachment of the metal to the apoenzyme. It is, therefore, significant that conditions have recently been found for dissociating the metal reversibly in the case of two zinc metalloenzymes, carboxypeptidase (58) and carbonic anhydrase (26), since this widens the range of experimental approaches available. In addition, with one group of metalloenzymes, namely, oxidative enzymes, it has sometimes been found possible to study the function of the metal ion while it is attached to the protein, as will be discussed in some detail in a later section.

A less clear situation is found in case of the term "metal-ion activation", which has been used to cover a great diversity of effects. First, it has been used misleadingly in cases where the metal ion can influence the reaction rate without the complex involved intrinsically participating in the catalytic mechanism. An example of such influences is the change in concentration of the reactive form of substrate or enzyme through competition between metal ions and protons; this effect is related to the well-known pH dependence of enzyme action. Often metal ions can also influence the reaction rate by stabilizing the active conformation state of the protein without really being essential for maintaining this structure. Since it is many times difficult to find stable conditions for the enzyme in the absence of the stabilizer, this effect can easily be mistaken for true activation. A good example of this is provided by recent work on α-amylases (51, 59), which contain several atoms of Ca per mole of enzyme. Stein and Fischer (51) were, however, able to show that only one Ca is essential for activity, but the enzyme in which the excess Ca has been removed is stable only in a very limited range of pH and temperature. If the conformation change resulting in a loss of activity is reversible, it is particularly difficult to distinguish between activation and stabilization. An example of this, not involving metal ions, is provided by the effect of some organic substances on the activity of carbonic anhydrase (7); with this enzyme all claimed activations disappear if the rate measurements are carried out at high enzyme concentrations, under which conditions the enzyme is stable. The stabilizing effects of metal ions will be considered further in connection with the subsequent discussion of the metal-ion activation of carnosinase.

The second and better use of the term "metal-ion activation" is to designate those cases where the metal ion directly partakes in the mechanism of the reaction, thus acting as a coenzyme. However, the coenzyme function also may involve different effects, distinguished on

[1] The original investigations of members of the Enzyme Chemistry Group reported in this communication have been supported by grants from the Swedish Natural Science Research Council and from the Division of General Medical Sciences, U. S. Public Health Service (RG-6542).

the basis of which complex (chelate) is functionally involved (34). First, the metal ion may function in a complex with the substrate, this metallosubstrate being the true substrate in the reaction. Secondly, a reversible binding to the protein may be involved; if only the metal-enzyme complex, but not the free protein, is catalytically active, such enzymes are distinguished from the true metalloenzymes only by their weaker interaction with the metal. Other influences, for example, on the velocity of breakdown of the enzyme-substrate complex [see (42)], are also possible.

Attempts to distinguish between the different mechanisms of activation just outlined have, in general, been based on kinetic measurements, but, as has been frequently pointed out (1, 34), a clear differentiation can seldom be made on kinetic grounds alone, since, under many conditions, identical rate equations are obtained. In the case of reversible reactions, where the activation can be studied both in the forward and in the reverse reaction, the kinetic approach is a more powerful discriminator, as pointed out by Griffiths *et al.* (14) and Wold and Ballou (63). However, their kinetic equations involve the concentration of free metal ions, so they still can be used only if knowledge of the binding of the activating ion to components in the reaction mixture is available.

It would appear, in fact, that the only rigorous approach for differentiating between the various types of metal-ion activation involves a study of the binding of metal ions to enzyme and to substrate, followed by a correlation of the thermodynamic data with kinetic measurements (34). To illustrate the experimental problems involved in such studies, I will describe briefly a few recent investigations carried out in the Enzyme Chemistry Group at Uppsala. I will, however, not go into details concerning the methods used for determining the stability constants, since they have been discussed by Dr. Gryder at this Symposium. The special problems in determining the binding of metal ions to proteins have been treated by Hughes and Klotz (18). In our work, we have used mainly pH titrations (38), an ion-exchange method (45) and metal indicators (6) for determining binding to substrates, while protein-metal interactions have been studied by equilibrium dialysis (18) with radioactive metals, estimated with a scintillation counter. In the case of Mn^{++}, an electron-spin resonance (ESR) method (36) has been found very convenient in the case of both substrate and enzyme complexes.

Among the metal-ion activated enzymes, peptidases assume a particular important position, since many of the hypotheses concerning the mechanism of action of the metal ion were first formulated for this group of enzymes, particularly by E. L. Smith (49). However, rigorous experimental tests of these ideas have been difficult due to the great instability of the enzymes, which has proved an obstacle both in purification and in kinetic work. Despite this complication, Smith has purified two metal-ion activated peptidases to an essentially homogeneous state (50), and, in Uppsala, Dr. Rosenberg (43) has prepared carnosinase as an apparently homogeneous protein. Dr. Rosenberg has also developed conditions under which purified carnosinase is stable for several months. This has made it possible for him to initiate an extensive series of investigations on the activating and stabilizing effects of metal ions with this peptidase (44).

To demonstrate that metal ions function as coenzymes in an enzyme reaction, it is necessary to show that their removal leads to a reversible loss of activity. However, such experiments are complicated in the case of carnosinase, since the metal-free enzyme is rapidly denatured. However, Dr. Rosenberg (44) has developed an electrodialysis technique by which the Mn^{++} used to stabilize the enzyme can be rapidly removed. The data in Figure 1 show that such dialysis inactivates the enzyme, but during the first few hours the activity is regained to about 90% on addition of Mn^{++}. However, as the metal-free enzyme is progressively denatured, the degree of reactivation also decreases (Fig. 1). While this experiment certainly is not sufficient to show that Mn^{++}, in addition to stabilizing the protein, acts as a coenzyme, Dr. Rosenberg has provided a very clear demonstration of this by a number of well-designed experiments (44), involving chelating agents and metal analyses, but space does not permit me to go into more details here. Mn^{++}, Cd^{++}, Zn^{++}, and Co^{++} have all been shown to activate carnosinase; Figure 2 illustrates the influence of the concentration of Mn^{++} and Cd^{++} on the activity.

While Mn^{++} can both stabilize and activate carnosinase, the two functions do not necessarily coincide. For example, Ca^{++}, which has no activating effects at all, is one of the best stabilizers, while Zn^{++}, although activating the enzyme, exhibits no stabilizing properties. As a consequence, the apparent metal-ion specificity is the result of a complicated superposition of the two

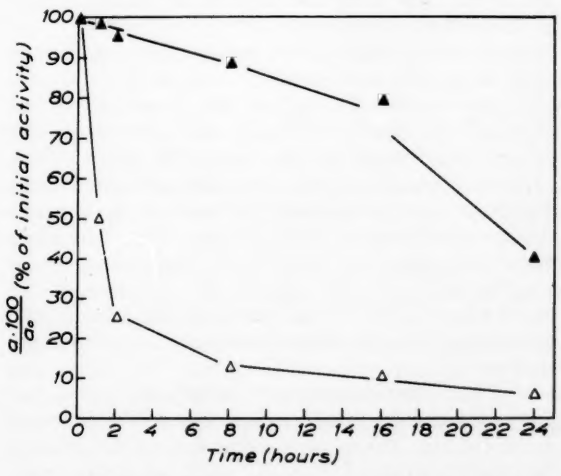


FIG. 1. Effect of electrodialysis on the activity of carnosinase. Two ml of a solution containing 0.01 M Mn^{++} dialyzed against tris-HCl buffer, pH 7.7, μ = 0.05; voltage: 0.9 v/cm; current: 35 mA. (△) residual activity; (▲) activity after reactivation with 2 x 10^{-3} M Mn^{++}.

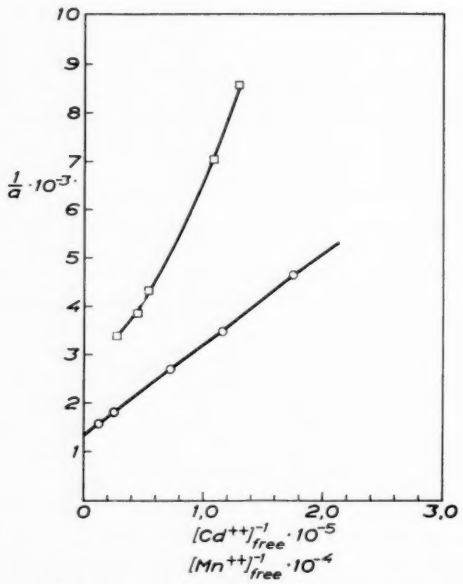


FIG. 2. Effect of the concentration of Mn^{++} and Cd^{++} on carnosinase activity, expressed in form of Lineweaver-Burk plots. □ Mn^{++}; ○ Cd^{++}.

effects and depends on the conditions of the activity measurements. Thus, the high metal-ion specificity previously attributed to carnosinase (17) is probably due to difficulties in obtaining a stable, metal-free enzyme and a failure to control secondary factors (such as denaturation) influencing the activity. The two-fold function of the metal ion can also explain the non-linearity of the Lineweaver-Burk plot with Mn^{++} (Fig. 2). The lag periods often observed with peptidases (48) have been shown not to be due to a slow step in the activation (37, 41) but are probably also related to the stabilizing properties of the metal ions.

Due to the instability of the metal-free enzyme, it has not been possible to investigate the binding of the activating ions to the protein, but the interaction of the substrate, carnosine, with Mn^{++} and Cd^{++} has been studied at two temperatures; the stability of the Cd^{++}-carnosine complex has also been measured by Martin and Edsall (39). A comparison between the thermodynamic and kinetic data indicates that activation involves the formation of a metal-enzyme complex; for example, the activation constant K_a (34) with Cd^{++} is $5 \cdot 10^{-6}$ M at 21°, while the values of pK_1 and pK_2 for the corresponding substrate chelate are 2.5 and 1.75, respectively.

With two other enzymes, enolase (31) and 3-phosphoglycerate kinase (3-PGK) (24), extensive measurements of the binding of activating metal ions to the proteins have been performed. 3-PGK binds metal ions very weakly, the dissociation constant in the case of Mn^{++} being $> 10^{-3}$ M (Fig. 3). Since activation occurs at a concentration of free Mn^{++} around 10^{-6} M, a metal-enzyme complex cannot be involved. Instead, a correlation of the kinetic data with the stability constants of the various substrate chelates shows that activation involves the formation of a Mn^{++}–ATP complex. Enolase, on the other hand, has one specific binding site interacting strongly with metals, and the active species is a metal-enzyme complex. The substrate also binds metal ions at high concentrations but this leads to inhibition by decreasing the concentration of free metal ions in equilibrium with the enzyme. Enolase has probably been more extensively studied than any other metal-enzyme complex, but the mechanism of metal-ion activation will not be discussed further here, since the present knowledge of this enzyme has recently been reviewed (31); however, some very recent data will be discussed in a later section of this communication.

While, as has been shown, the primary reaction of the activating ion may be either with the enzyme or with the substrate, the end result in all activation mechanism is usually assumed to be a ternary complex (34). A stimulating hypothesis has then been that, in this ternary complex, the metal ion acts as a link between enzyme and substrate (34, 49). Such bridge formation has been demonstrated in model systems, but a direct demonstration in the case of enzyme-substrate complexes is still lacking (16, 34). Since this problem has been reviewed in other places (16, 34), and also discussed by Dr. Eichhorn at this Symposium, it will not be considered further here.

FACTORS DETERMINING STABILITY OF METAL-ENZYME COMPLEXES

Studies on the binding of metal ions in metalloenzymes and metal-enzyme complexes have shown that there is a continuous spectrum in the strength of interaction. In an attempt to throw light on the different factors determining the stability of a complex between a metal ion and protein, we have initiated a comparative study of

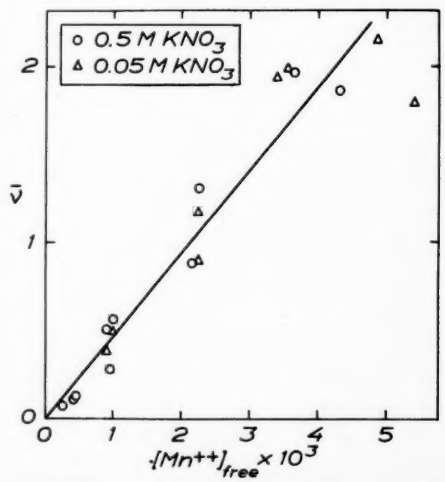


FIG. 3. Binding of Mn^{++} to 3-PGK in 0.05 M tris-HNO_3, pH 7.65, containing 0.5 M (○) and 0.05 M (△) KNO_3, respectively; temperature: c.38° C. $\bar{\nu}$ represents the average number of metal ions bound per molecule of protein.

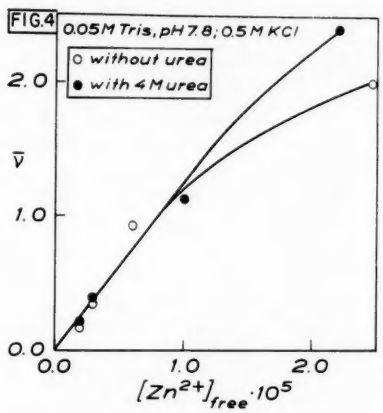


FIG. 4. Effect of urea on the binding of Zn^{++} to human serum albumin at pH 7.8 and 4° C. $\bar{\nu}$ represents the average number of metal ions bound per molecule of protein.

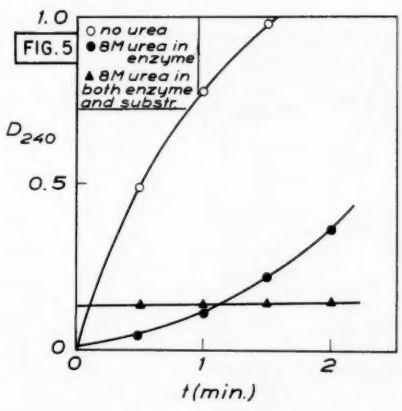


FIG. 5. Effect of 8 M urea on enolase activity. Substrate: 2.4×10^{-3} M DL-2-phosphoglyceric acid in tris-HCl, pH 7.4, μ = 0.05, containing 1.0×10^{-3} M Mg^{++}; 10 μl of enzyme (c. 1 mg/ml) added to 3 ml of substrate solution. D_{240} is a measure of product (phospho(enol) pyruvic acid) formed; temperature: 25° C.

the binding of Zn^{++} to human serum albumin, 3-PGK, enolase, carboxypeptidase and carbonic anhydrase. These proteins have been chosen because their metal complexes fall in different parts of the stability spectrum. Zn^{++} has been selected as the metal ion studied since this is a physiologically important ion (56) which functions in many true metalloenzymes as well as in several metal-ion activated enzymes. In addition, a convenient isotope (Zn^{65}) is available, facilitating the binding measurements, while many other physiologically active metal ions, such as Mg^{++}, form such weak complexes that accurate studies are difficult. With the exception of serum albumin, the Zn^{++} complexes of all the proteins studied are catalytically active.

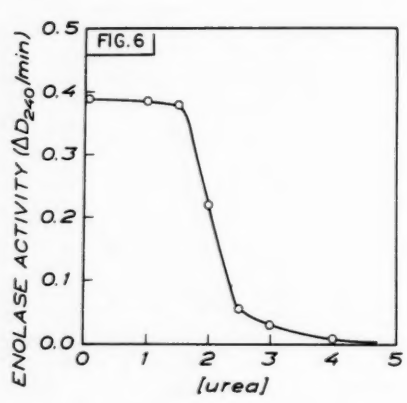
The results obtained so far in these investigations indicate that at least three different types of factors govern the stability of metal ion-protein complexes: 1. simple complex formation with polar groups in protein side chains (carboxyl, imidazole, etc.); 2. chelate formation with these same groups; and 3. indirect effect of protein structure, which may depend on (a) charge surrounding the binding site or (b) steric factors (metal "buried"). The accurate measurements of Gurd and Goodman (15) have shown that the first factor is sufficient to account for the Zn^{++}-binding to serum albumin. Binding to the imidazole groups of the 16 histidines present in the molecule dominates the interaction, and the calculated association constant agrees very well with that determined for Zn^{++}-imidazole complexes by Edsall *et al.* (12). 3-PGK binds Zn^{++} even more weakly than serum albumin, in agreement with its lower histidine content (M. Raznikiewicz, unpublished observations), and it would seem that simple complex formation is involved in this case also.

While the binding of Zn^{++} to enolase is sufficiently weak so that the metal can be removed by dialysis, earlier studies (28, 31) have shown that the protein has one specific binding site, whose interaction with the metal ion is much stronger than can be accounted for on the basis of simple complex formation with any protein side chains. Since the enzyme does not appear to contain any non-amino acid constituents (32), chelate formation was postulated (29) to account for the high affinity, and we have recently obtained strong experimental evidence for the correctness of this suggestion. As pointed out by Warner (61), denaturation of the protein should prevent chelate formation, while simple complex formation should be little affected. This prediction has been fully confirmed by a comparison of the effect of urea on Zn^{++}-binding to serum albumin and to enolase. Urea, in 4 M concentration, causes an unfolding of the secondary structure in serum albumin, as judged by a large decrease in optical solution ($[\alpha]_D$ changes from about $-50°$ to about $-90°$)[2], but this does not lead to any decrease in the binding of Zn^{++}, as seen in Figure 4[3]. In fact, at high concentrations of Zn^{++}, a slight increase in the binding can be noted; while Figure 4 contains rather few points, this increase is considerably larger than the experimental error, and it has now been observed in six similar experiments. It is probably due to the liberation of polar groups not available to the solvent in the native protein.

A quite different situation is found in the case of enolase. The denaturing effect of urea can here be followed not only by optical rotation (in 4 M urea, $[\alpha]_D$ decreases by about 45°) but also by activity measurements, since urea denaturation leads to a reversible loss of activity, as seen in Figure 5. The enzyme is almost completely inactivated in 4 M urea, as shown in Figure 6, and this concentration has been used in the metal-binding experiments. Figure 7 shows the effect of 4 M urea on the binding of Zn^{++} at the same pH (7.8) as used in the experiments with serum albumin. It can be seen that in this case denaturation causes a large decrease in binding, the difference approaching 0.75 Zn^{++} ions per mole of enzyme at high concentrations. Actually, slight variations in the degree of binding without urea, and in the difference between the "native" and denatured enzyme, have been observed from experiment to experiment, but these variations can be related to the specific activity of the

[2] The optical rotation data given here must be regarded as very approximate, as lack of a suitable instrument has prevented accurate measurements.

[3] The binding of Zn^{++} to urea was studied with the murexide method (6) and found to be negligible at the concentrations used in the dialysis experiments.



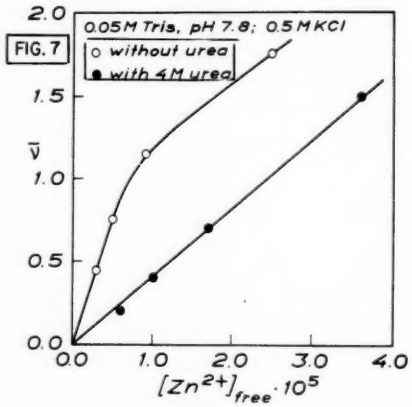


FIG. 6. Effect of the concentration of urea on enolase activity. Substrate: 2.4×10^{-3} M DL-2-phosphoglyceric acid in tris-HCl, pH 7.8, $\mu = 0.05$, containing 1.0×10^{-3} M Mg^{++}; 25 µl of enzyme (c. 1 mg/ml) added to 3 ml of substrate. The same urea concentration was present in both enzyme and substrate; temperature: 25° C.

FIG. 7. Effect of urea on the binding of Zn^{++} to enolase at pH 7.8 and 4° C. $\bar{\nu}$ represents the average number of metal ions bound per molecule of protein.

enzyme preparation used. Due to denaturing effects in various steps of the purification procedure (30), most preparations contain some inactive molecules; for example, the specific activity of the enzyme used in the experiment of Figure 7 indicates that only 80% of the protein present was active. Thus, the difference observed must be slightly increased, and it appears that urea destroys one binding site in the enolase molecule. This binding site, which has an affinity constant for Zn^{++} of about 10^{-5} M, is identical with the specific metal-binding site previously shown (28, 31) to be involved in enolase activation. The fact that its unusually high metal affinity is dependent on the secondary structure of the protein is a strong indication that chelate formation is involved, since it would appear difficult to explain the drastic effect of denaturation in any other way. The ligand groups are thus not present in adjacent amino acids in the sequence of the single peptide chain making up the enolase molecule (32), but they are brought together by the secondary folding of this chain.

Experiments with urea denaturation of carbonic anhydrase indicate that chelate formation is involved in the binding of Zn^{++} in this enzyme also. However, this is not sufficient to account for the experimental findings in this case, and indirect effects of the protein structure appear to influence the association between metal and protein. In Uppsala, Dr. Lindskog (25), using a method involving chromatography and zone electrophoresis, has purified bovine erythrocyte carbonic anhydrase, and Mr. Nyman (unpublished) has developed a similar purification procedure for the human enzyme. Both enzymes appear homogeneous by the common physical criteria. They differ somewhat in molecular weight (31,000 and 34,000, respectively, for the bovine and human enzyme) but careful analytical work has demonstrated the presence of 1.0 Zn^{++} per mole of protein in both cases (0.21 and 0.20% Zn, respectively). It was known already from previous work that the metal is very firmly attached to the protein, since electrodialysis does not remove it (46), and there is no exchange with radioactive Zn^{++} at neutral pH even during a period of a month (53). It has, therefore, generally been considered that the metal can only be removed on destruction of the protein [cf. Ref. (20)]. However, recently Dr. Lindskog (26), in collaboration with me, has shown that it is possible to dissociate the metal reversibly from the enzyme at pH 5, in the presence of chelating agents. The removal of Zn^{++} causes a loss of activity, which, however, can be fully regained by addition of Zn^{++}. Figure 8 shows that the activity of carbonic anhydrase that has been dialyzed extensively against 10^{-3} M 1,10-phenanthroline at pH 5 decreases to about 10% of the activity of the control but that addition of Zn^{++} causes a full restoration of activity. This reversible removal of the metal has also made it possible to test if other metals than Zn^{++} are effective in reactivating the enzyme. While a study of this question is still in progress, Dr. Lindskog has already shown that Co^{++} can give an activity which is about 50% of that with Zn^{++}, as shown in Figure 8; however, in this case the reactivation is considerably slower (Fig. 8).

Previous to our work, Dr. Vallee and co-workers (58) had found it possible to dissociate the metal reversibly in another Zn-metalloenzyme, carboxypeptidase, and it was, therefore, natural for us to compare the properties

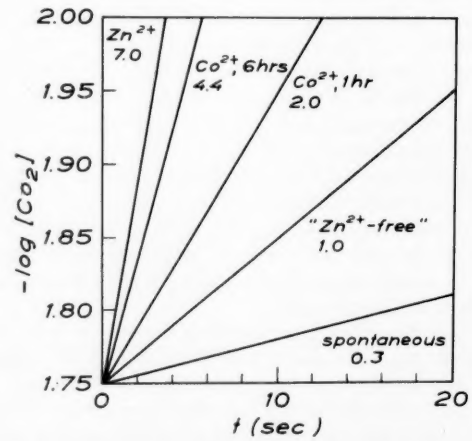


FIG. 8. Activity of carbonic anhydrase after dialysis against 10^{-3} M 1,10-phenanthroline for 30 days, and after reactivation of this enzyme with Zn^{++} and Co^{++}. For details of the activity measurements, see (25, 26); the figures given represent the slopes of the lines.

of the two enzymes in regard to their association with Zn^{++}. In equilibrium dialysis experiments with radioactive Zn^{++}, we showed that the affinity of carbonic anhydrase for Zn^{++} is very similar to that of carboxypeptidase, which Coleman and Vallee (8) have shown to have an apparent dissociation constant of 5×10^{-9} M. However, there is one major difference in the interaction of the two enzymes with the metal. While equilibrium in the case of carboxypeptidase is established as fast dialysis equilibrium, serveral weeks are required to remove the Zn^{++} from carbonic anhydrase, as shown for the human enzyme in Figure 9. Both human and bovine carbonic anhydrase exist in a number of electrophoretic forms, and the data in Figure 9 show that there is also a difference in the rate of dissociation between some of these forms. The slowness of the reactivation with Co^{++} has already been mentioned.

To explain the low rate of equilibration between protein and metal it appears necessary to assume that the metal is "buried" in the protein which then acts as a barrier to the diffusion of the metal ion. This could also explain why a low pH, in the absence of a chelating agent, is not sufficient to dissociate the metal, as in the case of carboxypeptidase (58), despite the similar dissociation constant: in the case of carbonic anhydrase, the function of the chelating agent is probably not mainly to displace the equilibrium but rather to increase the rate of diffusion of the metal ion by increasing the concentration gradient and, possibly also, by changing the charge. Further evidence for a steric effect of the protein comes from the lack of inhibition by many organic chelating agents. For example, 1,10-phenanthroline is a powerful inhibitor of carboxypeptidase and many other Zn enzymes (55), but it causes no decrease in carbonic anhydrase activity (10).[4] On the other hand, the small CN^- ion is a good carbonic anhydrase inhibitor. However, a number of facts indicate that a pure steric effect cannot be involved. First, CN^- is actually somewhat larger than Zn^{++}. Secondly, there is evidence that the Zn^{++} is located at the active site (11) to or from which OH^-, CO_2 and HCO_3^- must diffuse very rapidly, in view of the extremely high turnover number of the enzyme (9). It is instead suggested that, due to charge distribution around the active site, the protein acts as a barrier to cations but not to small anions or neutral molecules. While such an hypothesis would reconcile the equilibrium and kinetic data we now have available, a good deal of further experimentation is necessary for its verification. It does, however, appear definite from the experiments I have summarized here that chelation and indirect effects of the protein structure, rather than simple complex formation, play the major role in determining the association between proteins and metal ions in the metal enzymes.

ENZYMES WITH METALS IN SEVERAL VALENCE STATES

The types of experimental approaches which I have discussed so far, involving metal-binding studies under a variety of experimental conditions and correlations of such measurements with kinetic effects of the metal ions, can, as we have seen, decide which complex or chelate partakes in the catalytic mechanism, and they have also illuminated the factors determining the stability in metal-enzyme interactions. While such knowledge is essential for an understanding of the function of the metal ion in an enzyme reaction, the more interesting problem is really the part played by the various stereochemical and electronic influences of the metal in the molecular changes involved in the mechanism. It is obvious that, to yield clues to this question, the chelate studies must be extended beyond the determination of equilibrium constants.

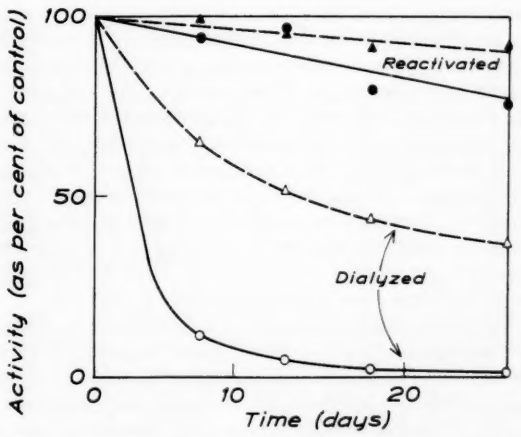


FIG. 9. Loss and regain of the activity of two forms of human carbonic anhydrase during dialysis at 4° C against 0.05 M acetate buffer, pH 5.0, containing 10^{-3} M 1,10-phenanthroline.

In regard to the detailed role of the metal in the reaction mechanism, a special position is occupied by redox enzymes containing metals that can exist in several valence states, since a natural hypothesis has been that the metal acts as an electron acceptor and donor, becoming alternately reduced and oxidized in the catalytic reaction. The metals involved in such enzymes are Fe, Cu and Mo, but I will not consider Fe enzymes, since the metal in these is usually heme-bound, while I am here mainly concerned with direct metal-protein interactions.

With Cu enzymes, in addition to teleological arguments and reasoning from analogies, a great number of experimental approaches have been used to determine the valence state of the metal in the resting enzyme and its possible change during the catalytic reaction, and here only a few examples can be given. Conclusions based on removal of the metal and reactivation by, for example, Cu^{++}, as in Kubowitz's (22) classical work on tyrosinase, are open to question since reducing groups in the protein may change the valency of the added metal. In fact, Kertész (21), using the specific Cu^+ reagent, 2,2′-biquinoline, claims that only Cu^+ is present in tyrosinase. However, it can equally well be argued that the presence of reducing groups in the enzyme, for example, −SH, lead to a false-positive reaction for Cu^+, and examples of

[4] Sturtevant (personal communication) has, in fact, observed an activation of carbonic anhydrase by 1,10-phenanthroline.

this have recently been described (13, 52). In view of these difficulties, it is fortunate that there are two physical methods available, namely magnetic susceptibility and electron-spin resonance (ESR), which allow the state of the metal ion to be followed during the reaction without any disturbance to the reaction system. Both of these methods depend on the paramagnetic properties of transition metal ions due to the presence of unpaired electrons. A change in the valence state of a metal will, in general, involve a change in the number of unpaired electrons; for example, Cu^{++} has one unpaired electron, while Cu^{+} has none, thus being diamagnetic.

In collaboration with Dr. Vänngård in the Physics Department at Uppsala, I have used ESR measurements to study a number of Cu enzymes. In fungal laccase, purified by Mr. Mosbach, we demonstrated the presence of Cu^{++} in the resting enzyme; on addition of excess substrate most of the metal is reduced to Cu^{+} but O_2 reoxidizes it (33). Nakamura (40), using the susceptibility technique, found the same behavior with the lacquer tree enzyme. In cooperation with Dr. Broman and Dr. Gedin, we have also found a similar situation in the copper protein of human plasma, ceruloplasmin. However, quantitative ESR measurements showed that the signal from the resting enzyme only accounted for about 50% of the Cu present according to analysis. While this would suggest the presence of an equimolar amount of Cu^{+}, it is possible that pairs of Cu^{++} in close vicinity could lead to a line-broadening making it impossible to detect the ESR signal. This question would probably best be resolved by quantitative susceptibility data, which are not yet available for this enzyme.

The discovery, around 1953, of metals in a number of flavoproteins caused a great interest in these enzymes, but as pointed out by Singer and Massey (47), the possible function of the metal in enzymic catalysis is quite problematic. In collaboration with Dr. Bray, we have studied one such enzyme, xanthine oxidase, and have shown that Mo changes valency during the reaction (5); Beinert and Sands (3) have demonstrated the same for Fe in DPNH-cytochrome c reductase. Since these studies have been discussed in two recent reviews (2, 60), they will not be considered further here. It should, however, be pointed out that all the ESR results presented so far must be interpreted with caution until correlations with kinetic measurements have shown that the changes observed occur in the main pathway of the electron-transfer reactions.

In addition to its use in studying the valence state, ESR can yield information on structure and bonding in complexes or chelates of transition metal ions. The main example of this approach in the metalloprotein field is the extensive studies of Ingram (19) on hemoglobin. Similar studies with Cu proteins have not yet been undertaken, partly because of the lack of sufficiently large crystals. However, Dr. Vänngård and I (35) have recently studied the ESR and visible spectra of a number of Cu proteins and model complexes, and our results allow some qualitative conclusions in regard to bonding. The proteins studied are laccase, ceruloplasmin, erythrocuprein and Cu^{++}-carboxypeptidase. The ESR spectra of frozen solutions of the proteins and other complexes all have the same general appearance, and, as an example, the spectrum of ceruloplasmin is shown in Figure 10. However, there are large differences in the so-called g-values and hyperfine structure constants, but space does not permit me to go into details concerning the experimental parameters used to characterize the spectra, and I will only briefly present some of our conclusions.

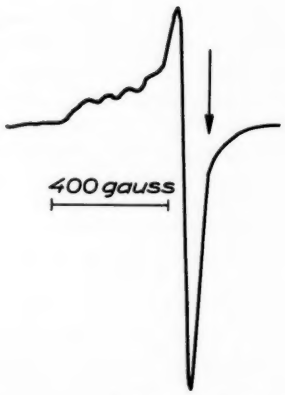


FIG. 10. ESR spectrum of a frozen solution of ceruloplasmin. The arrow indicates the resonance field for free electrons.

The most striking result seemed to us to be the fact that the two oxidative enzymes studied, laccase and ceruloplasmin, occupy a unique position, as apparent from their extremely small values of one of the hyperfine constants, which are a measure of the coupling between the unpaired electron and the nucleus, and from the values of parameters related to the contributions of σ- and π-bonding (62). These data indicate an unusual degree of delocalization of the unpaired electron in these enzymes, and it seems an attractive hypothesis that this is related to their oxidase function, since a high degree of delocalization of the unpaired hole on Cu^{++} should facilitate a mechanism involving a valency change in the metal.

The two nonoxidative proteins, on the other hand, show the same characteristics as model complexes having N as the ligand atoms, while O complexes show different properties. In addition, if the oxidative enzymes are denatured, they are transferred to the same category as the other proteins and, at the same time, their catalytic activity is lost. This shows again the importance of the secondary structure of the protein for the functional involvement of metals in enzyme catalysis.

CONCLUDING REMARKS

In the case of metal enzymes other than the redox enzymes just discussed, much less experimental information is available concerning the molecular function of the metal in catalysis. Various schemes have been advanced, resting mainly on analogies with model reactions and on considerations of the metal-ion specificity. Due to their hypothetical nature I will not discuss them here but

rather refer to a number of reviews already quoted (4, 34, 55, 57, 62). To obtain a firmer basis, it is obvious that new directions of experimentation are necessary, and I have already stressed that our studies must turn from an emphasis on stability constants to a determination of the structure and physical properties of the chelates involved in the enzymic reactions. Like the stability data, this information should then be correlated with kinetic measurements, particularly second-level kinetics (23). While beginnings in such studies have been made, they do not yet present a unified picture, and I will not attempt to summarize the results at this time.

As pointed out by Lumry (27), the course of future work suggested here seems, in fact, the only safe approach to the general problem of enzyme mechanism. It marks a slow road to progress in the study of the role of metal-binding in enzymic reactions, but I do not believe that a quicker route can be mapped out.

REFERENCES

1. ALBERTY, R. A. *Advances Enzymol.* 18: 1, 1956.
2. BEINERT, H. In: *The Enzymes* (2nd ed.), Vol. 2, edited by P.D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1960, p. 339.
3. BEINERT, H. AND R. H. SANDS. *Biochem. Biophys. Research Communs.* 1: 171, 1959.
4. BRAY, R. C. AND K. R. HARRAP. In: *Annual Reports on the Progress of Chemistry*, Vol. LV. London: The Chemical Society, 1959, p. 343.
5. BRAY, R. C., B. G. MALMSTRÖM AND T. VÄNNGÅRD. *Biochem. J.* 73: 193, 1959.
6. CHABEREK, S. AND A. E. MARTELL. *Organic Sequestering Agents.* New York: Wiley, 1959, p. 240.
7. CLARK, A. M. AND D. D. PERRIN. *Biochem. J.* 48: 495, 1951.
8. COLEMAN, J. E. AND B. L. VALLEE. *J. Biol. Chem.* 235: 390, 1960.
9. DAVIS, R. P. *J. Am. Chem. Soc.* 80: 5209, 1958.
10. DAVIS, R. P. *J. Am. Chem. Soc.* 81: 5674, 1959.
11. DAVIS, R. P. In: *The Enzymes* (2nd ed), Vol. 5, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1961, ch. 33.
12. EDSALL, J. T., G. FELSENFELD, D. S. GOODMAN AND F. R. N. GURD. *J. Am. Chem. Soc.* 76: 3054, 1954.
13. FELSENFELD, G. *Arch. Biochem. Biophys.* 87: 247, 1960.
14. GRIFFITHS, D. E., J. F. MORRISON AND A. H. ENNOR. *Biochem. J.* 65: 153, 1957.
15. GURD, F. R. N. AND D. S. GOODMAN. *J. Am. Chem. Soc.* 74: 670, 1952.
16. GURD, F. R. N. AND P. E. WILCOX. *Advances in Protein Chem.* 11: 311, 1956.
17. HANSON, H. T. AND E. L. SMITH. *J. Biol. Chem.* 179: 789, 1949.
18. HUGHES, T. R. AND I. M. KLOTZ. In: *Methods of Biochemical Analysis*, Vol. 3, edited by D. Glick. New York: Interscience, 1956, p. 265.
19. INGRAM, D. J. E. AND J. C. KENDREW. *Nature, London* 178: 905, 1956.
20. KELLER, H. AND U. H. PETERS. *Hoppe-Seyler's Ztschr. physiol. Chem.* 317: 228, 1959.
21. KERTÉSZ, D. *Nature, London* 180: 506, 1957.
22. KUBOWITZ, F. *Biochem. Ztschr.* 299: 32, 1938.
23. LAIDLER, K. J. *Discussions Faraday Soc.* 20: 83, 1955.
24. LARSSON-RAŹNIKIEWICS, M. AND B. G. MALMSTRÖM. *Arch. Biochem. Biophys.* 92: 94, 1961.
25. LINDSKOG, S. *Biochim. et biophys. acta* 39: 218, 1960.
26. LINDSKOG, S. AND B. G. MALMSTRÖM. *Biochem. Biophys. Research Communs.* 2: 213, 1960.
27. LUMRY, R. In: *The Enzymes* (2nd ed), Vol. 1, edited by P. D. Boyer, H. Lardy and K. Myrbäck, New York: Acad. Press, 1959, p. 157.
28. MALMSTRÖM, B. G. *Arch. Biochem. Biophys.* 46: 345, 1953.
29. MALMSTRÖM, B. G. *Arch. Biochem. Biophys.* 58: 381, 1955.
30. MALMSTRÖM, B. G. *Arch. Biochem. Biophys.* 70: 58, 1957.
31. MALMSTRÖM, B. G. In: *The Enzymes* (2nd ed), Vol. 5, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1961, ch. 29.
32. MALMSTRÖM, B. G., J. R. KIMMEL AND E. L. SMITH. *J. Biol. Chem.* 234: 1108, 1959.
33. MALMSTRÖM, B. G., R. MOSBACH AND T. VÄNNGÅRD. *Nature London* 183: 321, 1959.
34. MALMSTRÖM, B. G. AND A. ROSENBERG. *Advances Enzymol.* 21: 131, 1959.
35. MALMSTRÖM, B. G. AND T. VÄNNGÅRD. *J. Mol. Biol.* 2: 118, 1960.
36. MALMSTRÖM, B. G., T. VÄNNGÅRD AND M. LARSSON. *Biochim. et biophys. acta* 30: 1, 1958.
37. MALMSTRÖM, B. G. AND L. E. WESTLUND. *Arch. Biochem. Biophys.* 61: 186, 1956.
38. MARTELL, A. E. AND M. CALVIN. *Chemistry of Metal Chelate Compounds.* New York: Prentice-Hall, 1952, p. 78.
39. MARTIN, R. B. AND J. T. EDSALL. *J. Am. Chem. Soc.* 82: 1107, 1960.
40. NAKAMURA, T. *Biochim. et biophys. acta* 30: 640, 1958.
41. RABIN, B. R. In: *Metals and Enzyme Activity*, edited by E. M. Crook. London: Cambridge, 1958, p. 21.
42. RABIN, B. R. AND E. M. CROOK. *Biochim. et biophys. acta* 19: 550, 1956.
43. ROSENBERG, A. *Arch. Biochem. Biophys.* 88: 83, 1960.
44. ROSENBERG, A. *Biochim. et biophys. acta* 45: 297, 1960.
45. SCHUBERT, J. In: *Methods of Biochemical Analysis*, Vol. 3, edited by D. Glick. New York: Interscience, 1956, p. 247.
46. SCOTT, D. A. AND J. R. MENDIVE. *J. Biol. Chem.* 140: 445, 1941.
47. SINGER, T. P. AND V. MASSEY. *Record Chem. Progr. (Kresge-Hooker Sci. Lib.)* 18: 201, 1957.
48. SMITH, E. L. *Fed. Proc.* 8: 581, 1949.
49. SMITH, E. L. In: *Advances Enzymol.* 12: 191, 1951.
50. SMITH, E. L. AND D. H. SPACKMAN. *J. Biol. Chem.* 212: 271, 1955.
51. STEIN, E. A. AND E. H. FISCHER. *J. Biol. Chem.* 232: 867, 1958.
52. TAKEMORI, S., I. SEKUZU AND K. OKUNUKI. *Biochim. et biophys. acta* 38: 158, 1960.
53. TUPPER, R., R. W. E. WATTS AND A. WORMALL. *Biochem. J.* 50: 429, 1951.
54. VALLEE, B. L. *Advances in Protein Chem.* 10: 317, 1955.
55. VALLEE, B. L. In: *Proceedings of the IVth International Congress of Biochemistry (Vienna 1958). Symposium VIII: Proteins*, edited by H. Neurath and H. Tuppy. London: Pergamon, 1960, p. 138.
56. VALLEE, B. L. *Physiol. Rev.* 39: 443, 1959.
57. VALLEE, B. L. In: *The Enzymes* (2nd ed), Vol. 3, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1960, ch. 15.
58. VALLEE, B. L., J. A. RUPLEY, T. L. COOMBS AND H. NEURATH. *J. Biol. Chem.* 235: 64, 1960.
59. VALLEE, B. L., E. A. STEIN, W. N. SUMERWELL AND E. H. FISCHER. *J. Biol. Chem.* 234: 2901, 1959.
60. VÄNNGÅRD, T., R. C. BRAY, B. G. MALMSTRÖM AND R. PETTERSSON. In: *Symposium on Biological Free Radicals*, edited by M. S. Blois, Jr. New York: Acad. Press, 1961, p. 209.
61. WARNER, R. C. *Trans. N.Y. Acad. Sci.* 16: 182, 1954.
62. WILLIAMS, R. J. P. In: *The Enzymes* (2nd ed), Vol. 1, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 391.
63. WOLD, F. AND C. E. BALLOU. *J. Biol. Chem.* 227: 313, 1957.

DR. JOSEPH E. COLEMAN (Harvard Medical School): Dr. Malmström, in discussing the copper carboxypeptidase, did you mean to imply that the copper in this enzyme occupies the site normally occupied by zinc? If so, do you have analytical data?

DR. MALMSTRÖM: While we cannot be certain about this, we have some indications that this is true. The main one is that in equilibrium dialysis experiments, under conditions where Zn^{++} binds only to the active site, there is competition between Cu^{++} and Zn^{++}.

DR. COLEMAN: Does the copper replace radioactive zinc?

DR. MALMSTRÖM: As I said, the presence of Cu^{++} depresses the Zn^{++} binding, and, in addition, the sum of the bound forms of the two metals never exceeded one.

DR. COLEMAN: Does the zinc in your enzyme dissociate normally?

DR. MALMSTRÖM: Yes.

DR. FREDERIC L. HOCH (Harvard Medical School): I should like to ask Dr. Malmström about his remarks on the anomalous activation of carbonic anhydrase upon the addition of a chelating agent. Under special circumstances, this effect has been noticed with yeast alcohol dehydrogenase; for instance, Redetzki and Nowinski (Redetzki, H. E. and W. W. Nowinski. *Nature, London* 179: 1018, 1957) observed that low concentrations of 1,10-phenanthroline increased enzymatic activity while higher concentrations, of course, inhibit. This effect was proven to be the result of inhibitory metal contaminants, removed by addition of the ligand. Was your purified carbonic anhydrase analyzed for metals other than zinc, to eliminate such a possibility?

DR. MALMSTRÖM: My own first reaction to Sturtevant's report was also that removal of inhibiting metals could be involved. In addition to zinc, we have also analyzed for copper and iron, and we did, in fact, find a rather large amount of iron impurity. However, this could be removed by dialysis against 1,10-phenanthroline under conditions where zinc does not dissociate at an appreciable rate. We sent such a dialyzed enzyme, which by analysis had been found free of copper and iron, to Dr. Sturtevant, and he still found activation with this sample. I might also point out that we failed to observe the same activation under the conditions of our measurements. A possible explanation is that Sturtevant carries out his rate measurements at 25° C while we use 0° C, and the process may have a large temperature coefficient.

DR. HOCH: Analyses for only copper and iron, of course, cannot serve to eliminate the adventitious effects under discussion. In connection with such effects of low concentrations of chelating agents on metalloenzymes, I believe that the very long times required in your experiments to remove zinc from carbonic anhydrase through the action of 1,10-phenanthroline may have a similar basis. Much more rapid removal of zinc may be expected if sufficiently high concentrations of this and other metal-binding agents are used, and if the temperature is increased. The "cage" theory may not be necessary if threshold concentrations for the action of the ligand are exceeded.

DR. MALMSTRÖM: The point is, however, that in parallel Zn^{65} exchange experiments with carbonic anhydrase and carboxypeptidase, using exactly the same conditions (pH, ionic strength, temperature, etc.), there is a distinct difference in the rate while the equilibrium constants are almost the same. With carboxypeptidase equilibrium is reached in two days while about three weeks is required with carbonic anhydrase.

DR. WILLIAMS: I would like to ask a question about the electron-spin resonance method so that we should know at least what information can be obtained from it. First of all, I would like to ask Dr. Malmström what information one can deduce from the intensity of the signal. You seem to suggest that the intensity of the signal, that is, the height that you are blocking off, might be related to the amount of the cupric ion which is bound to a protein. However, at the same time, you said that in ceruloplasmin you made an estimate, and half was cuprous and half was cupric. What is the information one can get from the intensity? How broad is the spectrum? You really can't deduce how much of the cupric ion you have from that observation.

I think the next point is whether you can distinguish, by using the electron-spin resonance, to which site the copper is bound and whether, if you had a second site and the copper was adding on to this, you would get a summation.

DR. MALMSTRÖM: Admittedly, the measurement of intensities in ESR is a rather difficult problem. Absolute intensities usually are determined by comparison with a solution of known concentration, but the accuracy is probably seldom better than ±10 per cent, depending on the line width and intensity. The area under the absorption curve is integrated, and with broad lines it is difficult to take full account of the tails of the signal. In certain cases the signal even might not be detectable; for example, with most compounds of Fe^{++} and Co^{++}, where the significance of the absence of a signal would have to be considered from case to case. In such instances, a reduction of the line width is obtained sometimes when going down to low temperatures (4–20° K). On the other hand, ions such as Cu^{++} and Mn^{++} almost always have a relatively small line width, which is not particularly temperature dependent. The measured intensities, therefore, can be used confidently in such cases as a measure of the concentration.

In regard to the second question, I would like to refer to our article in the *Journal of Molecular Biology* (Malmström, B. G. and T. Vänngård. *J. Mol. Biol.* 2: 118, 1960) in which we showed that the different copper proteins and model complexes have resonance parameters (g-values and hyperfine structure constants) which are characteristic of the compound studied. As a consequence, if in a sample Cu^{++} was present bound in different ways, the ESR spectra, especially the hyperfine structure, would tend to be smeared out. Thus, when we get a well resolved hyperfine structure in the proteins, this is a good indication that all Cu^{++} ions are bound in the same way.

DR. HASTINGS: What is this electron-spin resonance? Can someone explain it in a few simple sentences?

DR. CALVIN: What you are measuring is the number of unpaired electrons which can flip back and forth in response to an oscillating field in an external magnetic field. The curve that Dr. Malmström had on the board is the differential of the absorption of energy from the oscillating field of a microwave beam that goes into the sample when the unpaired electrons are put in an external magnetic field. The microenvironment of these unpaired electrons depends on the nuclei around which they move. When Dr. Malmström spoke about nitrogen hyperfine structure, what he was describing was the micronuclear environment of the unpaired electron. When he spoke of nitrogen hyperfine structure on the copper unpaired electron, he meant that the shared electron of the

copper ion that he was looking at could be both on the copper nucleus and on the nitrogen nucleus. He could tell from a detailed analysis how much it sees the copper nucleus and how much it sees the nitrogen nucleus. This kind of analysis has been possible with the single crystals of dilute magnetic material. Whether it will be possible with the enzymatic materials remains to be seen. This is a very good example, the first one I have seen, of nitrogen hyperfine structure in an enzyme.

DR. ALBERT: Dr. Malmström had a lot to say about carbonic anhydrase on which he has worked a great deal. I was just wondering what the present opinion is on some post war German work which claimed that carbonic anhydrase has a dialyzable tripeptide which binds zinc. Has this work been substantiated?

DR. MALMSTRÖM: No, in fact, the discoverer himself has retracted this claim (Keller, H. *Hoppe-Seyler's Ztschr. physiol. Chem.* 320: 21, 1960). In a recent note (Keller, H., P. Gottwald and N. Wendling. *Biochem. Biophys. Research Communs.* 3: 24, 1960) he has presented some evidence, similar to that of Dr. Vallee in the case of carboxypeptidase, that a sulfhydryl group is involved in the zinc binding. Keller's peptide has been synthesized recently also and shown to bind Zn^{++} very weakly (Liefländer, M. *Hoppe-Seyler's Ztschr. physiol. Chem.* 320: 35, 1960).

DR. HARRY A. SAROFF (National Institutes of Health): I was glad that Dr. Malmström mentioned the electrostatic effects. Bovine serum albumin appears to exhibit a localized electrostatic effect on the binding of zinc to the imidazole groups. In measuring this effect, we started with the assumption the binding between zinc and perchlorate ions was small enough to be ignored. The activity of the zinc ion was found to be lowered by a factor of 3 when a 0.1 M nitrate solution was replaced by a thiocyanate solution. The charge on the albumin molecule was calculated by starting with an isoionic solution and measuring H^+, NO_3^-, and Zn^{++} binding after adjusting to pH 6 and adding nitrate and zinc ions. The same experiments were repeated with the thiocyanate ion in place of the nitrate ion. We found that the zinc ion bound to the protein with a constant in the presence of the thiocyanate ion that was about 30 to 40 times greater than that in the presence of nitrate ion. One explanation of this result is the presence of a localized electrostatic effect in the area of the protein molecule where the Zn^{++} binds.

DR. VALLEE: If I correctly understand the data from the ESR spectra on laccase, ceruloplasmin and copper carboxypeptidase shown here and published recently in the *Journal of Molecular Biology* (Malmström, B. G. and T. Vänngård. *J. Mol. Biol.* 2: 118, 1960), then the existence of a spectrum in the case of laccase and ceruloplasmin and its absence for copper carboxypeptidase implies to Dr. Malmström a difference in the nature of the binding groups. There is, however, the possibility of interpreting the data in an entirely different manner. I mentioned earlier that the SH group of the carboxypeptidase prepared from pancreatic juice according to the method of Anson (Anson, M. L. *J. Gen. Physiol.* 20: 663, 1937) oxidizes very quickly upon removal of zinc from this enzyme. With this enzyme, we have been unable, in fact, to demonstrate binding of zinc and other metals with concomitant restoration of activity. In contrast, the enzyme prepared by the unpublished method of Allan, Keller and Neurath from pancreas acetone powder oxidizes much more slowly upon removal of the zinc; thus, metal restoration is feasible. Since the enzyme prepared according to Anson's method was employed here, in the absence of analytical data, it cannot be assumed that copper is bound without analytical confirmation of this fact. Thus, the absence of an ESR spectrum can mean simply that copper was not bound to apocarboxypeptidase.

DR. MALMSTRÖM: I don't think that you understand correctly, Dr. Vallee, since it is not a question of the absence of a spectrum in carboxypeptidase, but only that its spectrum differs from those of the oxidases. In addition to Cu^{++} carboxypeptidase we also had a naturally occurring Cu protein without oxidase activity, namely erythrocuprein, which gave an ESR spectrum very similar to that of Cu^{++} carboxypeptidase but different from the spectra of the two oxidases. There is no doubt that Cu^{++} is bound to the protein in the case of carboxypeptidase, and the shape of the spectrum indicates that all Cu^{++} ions are bound to the same kind of sites. We cannot, however, be certain that this is the zinc binding site, but, as I have mentioned earlier in the discussion, we have evidence which points in this direction. The difference compared to the oxidases does not concern, therefore, the question of whether the Cu^{++} is bound or not but rather its chemical environment and mode of binding, which in the oxidases must be different from that in the models and other proteins.

DR. SCHEINBERG: I have just learned everything I know about electron-spin resonance from Dr. Calvin. I gather from his description of it that whether the copper is bound in the protein to sulfur, nitrogen or oxygen will affect the structure one sees in electron-spin resonance. I think it is relevant to the point that Dr. Vallee has just made about the difference in the ESR studies between the two enzymes that there is some evidence, which Dr. Aisen obtained about two years ago, that in ceruloplasmin the copper is probably bound, at least on one site, to a carboxyl group. There is Dr. Vallee's evidence that the copper in carboxypeptidase is bound to a sulfhydryl. So, Dr. Calvin, could this be the basis of the explanation of the difference in ESR results of the two proteins?

DR. CALVIN: That amounts to what I was trying to say. The objection I was going to raise was to Dr. Vallee's interpretation that the difference between these various spectra implied that in one of them the copper was not bound and in the other it was bound. Different environments, that's all.

DR. VALLEE: I merely wanted to point out that the absence of an ESR spectrum cannot be interpreted as a positive, meaningful finding when data are not existent proving that copper was bound to apocarboxypeptidase. Such a negative result would have meaning only if a copper-protein complex can be verified analytically.

DR. MALMSTRÖM: But I have not talked about any absence of an ESR spectrum, only about differences in the resonance parameters.

DR. WILLIAMS: In joint conversation with Drs. Malmström and Scheinberg the following conclusion has been reached. The blue color of copper proteins cannot be related to the valence state of copper except perhaps in the following cases: *1*) erythrocuprein where the extinction coefficient and wave length, around 250 at 670 mμ, suggests cupric, *2*) hemocyanin where the extinction coefficient is approximately 10,000 and where the valence state of copper is known to be cuprous. In no other case can we certainly ascribe the blue color to either valence state. If analogy between the two known cases, using as well Dr. Malmström's data on ESR and magnetic susceptibility results in the literature, is permitted, however, we must conclude that the copper proteins which are intensely blue, extinction coefficient from 1,000 to 10,000, contain from 10% to 100% cuprous. Tyrosinase is then a cupric complex while all others appear to be equilibrium mixtures of cuprous and cupric complexes. Amongst models intensely blue cuprous complexes are known but no intensely blue cupric complexes.

Summary remarks by the chairman

ARTHUR E. MARTELL

THE LECTURES given in this section of the symposium have been directed at an understanding of the nature of the coordination compounds of metal ions, and at an interpretative description of their physicochemical behavior.

We have seen that the nature of bonding between ligand and metal can be understood from the point of view of the electrostatic concept of coordination and the principles involved in the chelate effect. For the less basic metals, the binding is more covalent in nature, as is indicated by thermodynamic studies of complex equilibria. For the transition metals, an important contribution to the nature of bonding and to chemical reactivity has recently been made through the development of the ligand field theory. Further development in the area of coordinating tendencies of metal ions and ligands requires extensive studies of the thermodynamics of reactions involving coordination compounds—with the purpose of determining heats and entropies of interaction. Such data are now available for only a relatively few systems.

One of the results of the now voluminous stability data in the literature is the ability to describe precisely, under restricted conditions, the variation of metal ion activities with pH and concentrations of various ligands. Extension of this approach to the wide variation of conditions found in biological systems requires much more information about the influence of ionic strength, nature of the electrolyte, and of charges of ligand and metal ion on their activities in solution. These factors are not well understood, with the result that equilibrium data in the literature cannot be readily converted to conditions very much different from the experimental conditions.

The kind of information which now seems to be needed most, and about which we seem to have the least information, is a knowledge of the microscopic structures and affinities of individual groups in a ligand for various metal ions. It is this kind of information which indicates specifically which of a number of alternative donor groups in a ligand actually combine with a metal ion. Thermodynamic data alone can never give this kind of information. The determination of specific reaction groups and affinities has been carried out in relatively few cases (see, however, the publications of J. Edsall and co-workers). In order to obtain such microscopic information on metal complexes and chelates, it will be necessary to apply every available experimental technique of the type that give information about structures: infrared and ultraviolet spectra, optical activity, isomerism, optical rotatory dispersion, and NMR and EMR spectra where they apply. We have seen the beginnings of this type of research in some of the papers presented in this section—such as, for example, the use of ESR described by Dr. Malmström, the application of rotatory dispersion as reported by Dr. Eichhorn, and the masking of specific groups before potentiometric titration, explained by Dr. Martin. Only through techniques such as these will it be possible to obtain precise microscopic information on metal-binding by small molecules,—the most useful for the purpose of deducing the nature of metal-binding by the more complex ligands found in biological systems.

In conclusion, I want to thank the speakers of this session for covering so well, and with so little overlap, such a large amount and wide variety of information on recent advances and interpretations in the field of metal complexes and chelate compounds.

II. PHYSIOLOGICAL ASPECTS OF METALS

GEORGE C. COTZIAS, *Chairman*

The "active catalytic site," an approach through metalloenzymes[1]

BERT L. VALLEE

Biophysics Research Laboratory, Department of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Massachusetts

IN RECENT YEARS a great deal of effort has been devoted to the definition of the role of metals in enzymatic catalysis (18, 53). Progress in this field has been aided greatly by the establishment of operational criteria for the isolation and identification of metalloenzymes (34, 36).

Historically, the inhibition of enzymes by metal complexing agents has served as the "classical" means of establishing the essentiality of a metal in the catalytic process (51). The many, newly synthesized chelating agents now used for this purpose have expanded this most important aspect of the field. The principles which govern their action have been discussed amply and do not require detailed recapitulation (34, 36, 53, 54). Concisely stated, such chelating agents may inhibit catalytic activity through specific interaction with the metal by removing it from the apoenzyme, through the formation of a mixed complex consisting of an enzyme-metal-chelate or through the formation of an enzyme-metal-substrate-inhibitor complex. Insight concerning the modes of the interactions may be gained through standard inhibition kinetics which serve to delineate the reversibility or preventability of such inhibitions and to determine the competition between chelating agents and substrates or coenzymes for an active metal site (36).

Much of this work has been concerned with a decision as to whether or not a metal present in an enzyme is indispensable for its activity (18). Inhibition may be *consistent* with the presence of a metal, but inhibition alone cannot serve to identify it. It must be stressed, moreover, that the functional effects of chelating agents cannot substitute for inorganic analysis. Studies correlating changes in function of an enzyme with its metal content while proving the essential role of the metal in enzymatic catalysis do not determine *how* the interaction of a protein with a metal brings about the activity and the exquisite specificity which is characteristic of enzymes.

The earliest views of the chemical mechanisms of enzyme action, at the beginning of the century, were derived mainly from the study of iron metalloenzymes, since they were best studied at that time. The enzyme was regarded as a "colloidal substance" owing its activity to a noncolloidal, iron-containing prosthetic group. The colloidal carrier, later recognized to be a protein, was thought to be nonspecific serving primarily to stabilize the prosthetic group (56). The importance of protein structure and composition in the events of catalysis is now well recognized and much effort has been directed toward modifying enzymes by physical and chemical means, in order to discern the properties of the protein molecule required for activity. These studies, while documenting the role of the protein, have actually reinforced the initial concept that only a small part of the enzyme molecule is involved in the catalytic process, since segments of some enzymes can be removed or altered without affecting activity. Current ideas hold that only relatively small part of the molecule must be arranged in critical configurations to result in activity: an "active catalytic site" (19). At this point it is germane to ask: what structural, configurational and compositional features of protein molecules endow them with enzymatic function? The presence of a metal atom as a component of the enzyme raises the question in even more specific terms. What are the features of the apoenzyme which, in conjunction with the metal atom, endow their joint product with catalytic specificity, a property exhibited neither by the protein nor the metal alone? Answers to these questions have been sought through

[1] The original work discussed in this paper was supported by grants-in-aid from the National Institutes of Health of the Department of Health, Education and Welfare, No. H3117 (C1) and by the Howard Hughes Medical Institute.

modifications of the primary, secondary and tertiary structure of enzyme molecules in order to delineate the exact composition and configuration of active catalytic sites of enzymes.

Chemically selective reagents have been employed to bring about inhibition of enzymatic activity through specific interactions with the reactive groups of amino acid residues. This approach has encountered difficulties since few such reagents have been found which are sufficiently specific. Moreover, the reactivity of amino acid side chains with such reagents may vary widely between chemically identical residues within a single protein, and even more so between different proteins. Further complications arise since it may be necessary to detect one or two "active" polar groups among many chemically identical but functionally inert groups. To be meaningful, studies depending on inhibition by selective end-group reagents, must be validated by tedious and difficult analytical characterization of the resultant protein derivative. Because of the dangers that imperfect selectivity, denaturation, or changes in charge or solubility of the enzyme may be interpreted fallaciously to indicate a specific chemical modification of an amino-acid end group with functional consequents, great caution in the interpretation of such experiments has been advised (11).

Highly purified metalloenzymes obviate some of the problems encountered with enzymes of purely organic composition. The physical properties and chemical reactivities of metal ions, firmly incorporated into the structure of native enzymes, differ *in kind* from the remaining organic segment of the protein molecule. Different avenues of approach are thus offered for the delineation of active catalytic sites in metalloenzymes.

Isotopic labeling of the metal atom facilitates its localization and identification within the protein. Chelating agents, reacting with metals, but not with polar side chains of the protein, meet the stringent dual requirements of selectivity and mildness, demanded for reagents used in the investigation of groups essential to enzyme activity. A most important dimension is added to these studies when the removal of a metal from a metalloenzyme—with concomitant loss of activity—is fully reversible. This renders feasible the demonstration of the reactive groups of the uncovered metal-binding site by the differential reactivity of selective end group reagents with the metal-containing and metal-free enzyme. This procedure has been employed successfully with carboxypeptidase A (*vide infra*) (39). Metals may thus be included in the list of reactive enzymatic loci which can be studied by means of specific and selective reagents (36).

Since zinc enzymes have been the specific objectives of our studies, this discussion will be limited to the properties of some of these. By the operational definitions of metalloenzymes, a total of nine zinc enzymes have been identified and characterized, eight of them since 1954: carbonic anhydrase of bovine erythrocytes (17), carboxypeptidase A of bovine pancreas (43), alcohol dehydrogenase of yeast (41) and horse liver (31, 42), glutamic dehydrogenase of beef liver (37), lactic dehydrogenase of rabbit skeletal muscle (49), alkaline phosphatase of porcine kidney (20), alkaline phosphatase of *E. coli* (23), and carboxypeptidase B of porcine pancreas (10).

In addition, procarboxypeptidase A from bovine pancreas, the zymogen of carboxypeptidase A, contains stoichiometric quantities of the metal (45). A calcium metalloenzyme, α-amylase of *B. subtilis*, has also been shown to contain zinc, although its presence does not seem thus far to have functional implications (48).

The sequential discovery of these enzymes, heterogeneous in catalytic specificity and previously unknown to contain this metal, is telling evidence of the usefulness of the operational approach (34, 35).

In the exploration of the active centers of zinc enzymes we have concentrated our efforts on yeast alcohol dehydrogenase, the analogous enzyme from horse liver, and on carboxypeptidase A of bovine pancreas. The native enzymes contain stoichiometric quantities of zinc which is essential for catalytic activity, as is readily apparent by their inhibition with chelating agents. Since the precise role of the zinc atoms in these three enzymes, however, is quite different, it would seem profitable to review some of the recent evidence concerning the specific action of zinc in these systems.

ALCOHOL DEHYDROGENASE OF YEAST[2]

The molecular weight of the crystalline enzyme is 150,000, it binds 4 molecules of diphosphopyridine nucleotide (DPN) and contains 4 atoms of zinc per molecule (41). Yeast, grown in the presence of Zn^{65}, biosynthetically incorporates 4 gram atoms of zinc to form $[(YADH)_4Zn_4^{65}]$. Under the conditions employed, these biosynthetically incorporated zinc atoms were not miscible with zinc present from extraneous sources (35).[3] Rapid loss of activity, proportional to the removal of zinc, follows exposure of the enzyme to pH levels below 6. A large number of chelating and complexing agents capable of combining with zinc inhibit it: 1,10-phenanthroline, α,α'dipyridyl, 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, diphenylthiocarbazone, thiourea, sodium diethyldithiocarbamate, 2,3-dimercaptopropanol, ammonium-N-nitrosophenylhydroxylamine,

[2] The following abbreviations will be employed (in formulations and when required for differentiation): $[(YADH)_4Zn_4]$, apoalcohol-dehydrogenase, where (YADH) represents the subunit of molecular weight 36,000; $[(YADH)_4Zn_4](DPN)_4$, the active holoenzyme, the brackets indicate the firm binding of zinc; [(CPD)Zn], zinc carboxypeptidase, (CPD) represents the apoenzyme; other metals may be substituted for zinc, i.e. [(CPD)Co]; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-dL-phenyl lactate; OP, 1,10-phenanthroline.

[3] Alcohol dehydrogenase, crystallized from horse liver, although it catalyzes the same reaction, differs in many respects from the yeast enzyme. The molecular weight is 73,000 or 84,000 (7), it binds two DPN molecules at neutral pH values and contains 2 gram atoms of zinc (42). Enzymatically it is less specific than the yeast enzyme, oxidizing vitamin A_1 alcohol and glycerol, as well as ethanol. Like the yeast enzyme it loses zinc and activity *pari passu* upon dialysis at pH values below 5.5 (35).

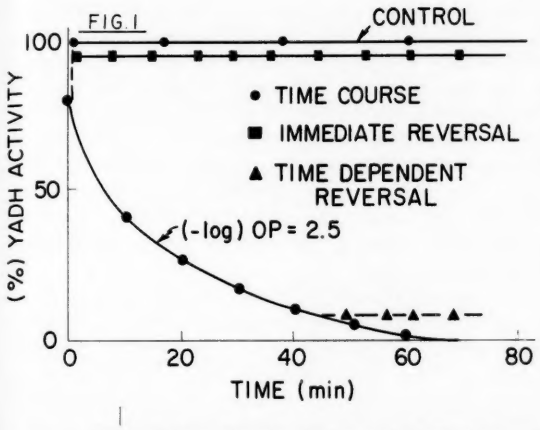


FIG. 1. Effect of time of preincubation and of dilution on the inhibition of yeast alcohol dehydrogenase by 1,10-phenanthroline. Conditions: the enzyme is exposed to the indicated concentration of the chelating agent at pH 7.5, 0°; aliquots are removed from the mixture at the times noted by experimental points. Assay as in (41). The inhibited enzyme (●) is diluted as soon as experimentally practicable (■) demonstrating reversibility. Irreversibility is seen after prolonged exposure (▲).

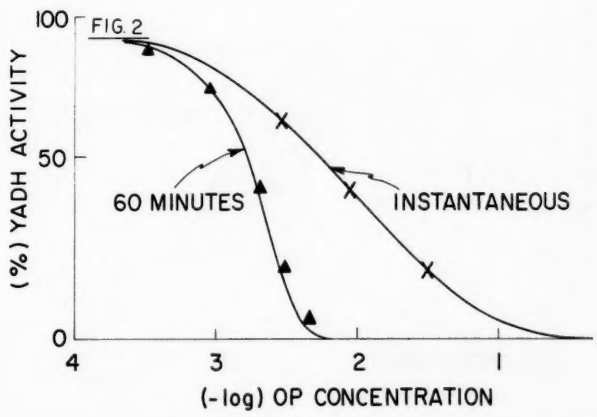


FIG. 2. Effect of 1,10-phenanthroline concentration on yeast alcohol dehydrogenase activity. Instantaneous inhibition (×); after 60 minutes (▲); the marked increase in the slope of the time-dependent inhibition curve is apparent and corresponds to the irreversibility of the changes observed.

cupferron, thiosemicarbazide, sodium sulfide, potassium cyanide, sodium azide, 4,7-dihydroxy-1,10-phenanthroline, thyroxine, quinaldinic acid, titan yellow, *p*-nitrobenzene-azoresorcinol, quinalizarin, imidazole, N-methylnicotinamide, 8-aminoquinoline, kojic acid, butazolidine, aneurin disulfide, 3,4-dithiotoluol, and tetraethylthiuram disulfide (36). The degree of inhibition is dependent on the concentration of these chelating agents, the duration of their contact with the enzyme prior to measurement of activity, and on the pH and temperature at which they are pre-incubated. The enzyme is also inhibited by traces of cupric, silver, ferrous, cadmium, mercuric ions, and also by zinc ions, the very metal which is an integral, functional part of the apoenzyme molecule. Additions of low concentrations of certain chelating agents may increase activity although higher concentrations of the same agents inhibit. This apparent paradox was resolved when it was demonstrated that this activation is due to the removal of traces of inhibiting metals—introduced with impure water—by the chelating agent (24).

In our continuing efforts to correlate function with composition and structure of yeast alcohol dehydrogenase we have employed the specificity of attack upon zinc of one of these agents. The inhibition kinetics indicate (14, 55) that 1,10-phenanthroline (OP) affects the activity of yeast alcohol dehydrogenase in two distinct steps:

$$\text{YADH} \cdot \text{Zn} + \text{OP} \underset{}{\overset{K_I}{\rightleftharpoons}} \text{YADH} \cdot \text{Zn} \cdot \text{OP} \qquad (1)$$

$$\text{YADH} \cdot \text{Zn} \cdot \text{OP} + \text{OP} \xrightarrow{k} \text{YADH} \cdot \text{Zn} \cdot (\text{OP})_2 \qquad (2)$$

where YADH·Zn represents each zinc atom of $[(\text{YADH})_4\text{Zn}_4]$ as an independent active site.

The first step is the instantaneous formation of a dissociable, mixed enzyme-inhibitor complex, consisting of one molecule of 1,10-phenanthroline bound to each zinc atom. The second step is irreversible in an enzymatic sense, and is consistent with the attachment, as a function of time, of a second molecule of 1,10-phenanthroline to the dissociable enzyme-inhibitor complex. Since the binding of 1,10-phenanthroline to zinc ions is completely reversible, the second step was thought to involve irreversible changes in protein structure (13). The type of inhibition data on which such deductions are based are summarized in Figures 1–3 which should be inspected sequentially since they are designed to give an over-all view of the reactions. Both the instantaneous, reversible and the time-dependent, irreversible inhibitions are shown in Figure 1. Figure 2 demonstrates the dependence on inhibitor concentration of the two reactions. The interdependence both on time and on inhibitor concentration are graphically integrated in Figure 3 from which the inhibition may be predicted for any given combination of these parameters.

This reaction sequence derived from inhibition kinetics, has been verified chemically. The absorption spectrum, characteristic of $[\text{Zn OP}]^{++}$ complexes, is also observed when yeast alcohol dehydrogenase is exposed to OP (40). A rise in extinction of the absorption maximum with time corresponds to the binding of the second OP molecule, followed by the removal of the metal from the molecule (*vida infra*).

The active role of the zinc atoms of liver alcohol dehydrogenase is also demonstrated by the inhibiting effects of metal-binding agents (42), but a difference exists in the interaction of 1,10-phenanthroline with the horse liver and the yeast alcohol dehydrogenases. While the yeast enzyme exhibits both the immediate and the time-dependent inhibition with 1,10-phenanthroline (14, 55), with the liver enzyme—under identical conditions—only the first, instantaneously reversible reaction

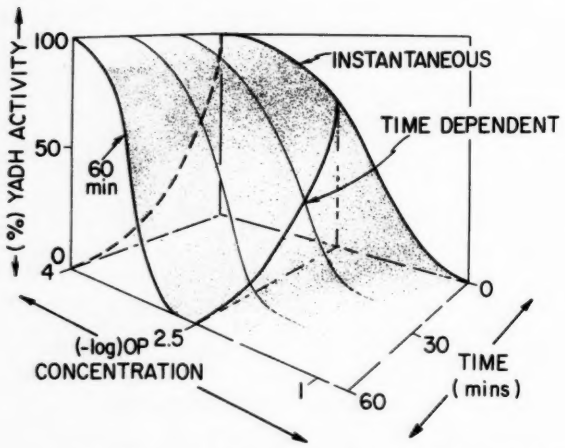


FIG. 3. Activity surface incorporating the dependence of the inhibition of yeast alcohol dehydrogenase on the concentration of 1,10-phenanthroline (abscissa) and on the time of contact with it (receding axis). The cross sections of the surface indicate the time-dependence of the phenomenon at one single concentration of the inhibitor (see Fig. 1). The inhibition at a concentration of $-(\log)$ OP 2.5, indicated by the solid black line, is projected onto the 10^{-4} M plane of inhibitor concentration, the dashed line. The plane of the surface itself indicates the progression of inhibition from instantaneous to that observed at 60 minutes (see Fig. 2). Inhibition, consisting of mixtures of both instantaneous and time-dependent inhibitions, is predictable and experimentally confirmed over the interval of 1,10-phenanthroline concentrations and times of exposure.

is observed (42). The inhibition kinetics indicate that one molecule of 1,10-phenanthroline reacts with each zinc atom to produce inactivation through the formation of a complex containing 1 mole of this inhibitor per each zinc atom of the liver enzyme (50), a mechanism which was again verified spectrophotometrically (38). The dissociation constant of this complex, determined both spectrophotometrically and kinetically, is of the order of 10^{-5} M, much lower than that of yeast alcohol dehydrogenase (1.5×10^{-3} M), and approaching that observed with free zinc ions and 1,10-phenanthroline, 10^{-6} M (35).

The instantaneous, reversible interaction of 1,10-phenanthroline with the zinc atoms of yeast or liver alcohol dehydrogenase is competitive both with DPN and DPNH, but is *not* competitive with the substrates, alcohol and acetaldehyde. The mechanisms of action and the reaction sequences, based on these inhibition kinetics have been detailed (13, 14, 55). Each zinc atom is thought to be involved in coenzyme binding, but not in substrate binding.

Further extensions of the interrelationships of composition, and function and their implications to the configurational properties of the active center of metalloenzymes derive from studies of optical rotation and rotatory dispersion, a physical approach particularly well suited to this purpose (6, 27, 52).

The optical asymmetry of many metal chelates as well as proteins suggested that these stereochemical properties might yield direct information on the conformation of the active catalytic center of yeast alcohol dehydrogenase Such studies of enzyme-metal-chelate complexes might be expected to permit both the joint examination of the simultaneous contributions to optical rotation made by the protein and the metal chelate, and the segregation of these contributions into two discrete components. This expectation is borne out experimentally, thus supplementing and integrating the views of the molecular configuration, activity, and composition of the activity center of the enzyme.

The time-dependent inhibition of yeast alcohol dehydrogenase by 1,10-phenanthroline is directly correlated to changes in specific optical rotation (Fig. 4). The formation of the enzymatically inactive enzyme-zinc-chelate complex results in a change of $[\alpha]_{546}$ from $-40°$ to $-55°$. Changes in secondary structure, as seen in denaturation, are not observed as evidenced by the unaltered dispersion constant, λc, measured between 365 and 600 mμ (32). The optical rotatory dispersion in the visible spectrum thus implies that 1,10-phenanthroline inhibition proceeds through a mechanism which does not involve a change in the conformation of the protein. Therefore, the superimposition of the zinc phenanthrolinate chromophore onto the background rotation of the protein was postulated to account for the observed changes in specific optical rotation. The observation of anomalous rotatory dispersion at 290 mμ, the wavelength of maximum absorption of the zinc phenanthrolinate chromophore, represents confirmation of this hypothesis (Fig. 5) (33). The competition of DPN, previously stressed in studies of activity and also seen in the observations on structural changes (*vide infra*), are again observed (32). This approach provides a striking potential to be used in resolving the mechanism of action of this and similar enzymes; and, moreover, it serves as a general method for the elucidation of the contribution of the protein and of the metal chelate to the total optical rotation in protein-metal-chelate-systems.

All of these studies of the role which the zinc atom may play have implied that it serves as a primary, active, enzymatic site. New evidence suggests, however, that the metal also participates in the maintenance of the *structural* integrity of yeast alcohol dehydrogenase (15, 16, 35).

As a function of the time of exposure and of the concentration of chelating agents, particularly 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, the apoalcoholdehydrogenase of molecular weight 151,000 dissociates into four equal subunits of 36,000, while the four zinc atoms are removed concomitantly. The depolymerization is correlated directly to the time-dependent inhibition of the molecule and is therefore equivalent to the binding of a second molecule of 1,10-phenanthroline (16) (Fig. 6). The time-dependent inhibition had previously been postulated to represent an alteration in the structure of the apoenzyme (13), an hypothesis which is now confirmed. These data extend the tetrad stoichiometry of the zinc atoms and DPN binding sites to the

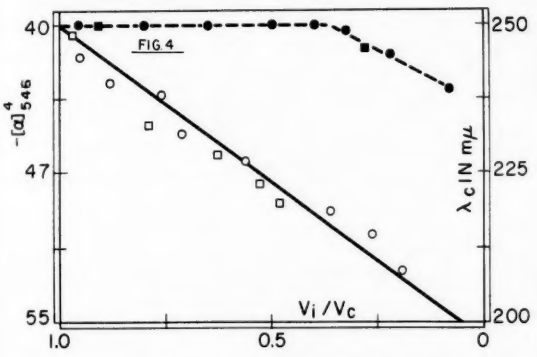


FIG. 4. Effect of chelating agents on the optical rotation of yeast alcohol dehydrogenase. Specific rotation, $[\alpha]_{546}$, —— and the dispersion constant, λc, ----- are plotted versus partial velocity, vi/vc. The change of specific rotation correlates with the loss of catalytic activity while the dispersion constant is unaltered until inhibition is nearly complete. Conditions: yeast alcohol dehydrogenase 5 mg per ml in 0.1 M phosphate, pH 7.5, 4°; measurements of $[\alpha]_{546}$ in the presence of 5×10^{-3} M 1,10-phenanthroline, (○), of λc, (●), and measurements of $[\alpha]_{546}$ in the presence of 2×10^{-2} M 8-hydroxyquinoline-5-sulfonic acid, (□), of λc, (■). Rotations determined in jacketed polarimeter cells of 10 cm path length. (See Ref. 33.)

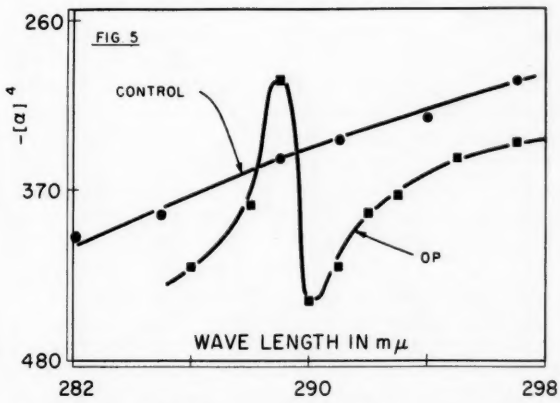


FIG. 5. Anomalous dispersion of yeast alcohol dehydrogenase-zinc-OP chromophore: specific rotation, $[\alpha]$, is plotted versus wavelength. The negative "Cotton effect" curve has a peak at 290 mμ and a trough at 289 mμ. 5 mg per ml yeast alcohol dehydrogenase in 0.1 M phosphate, pH 7.5, 4°, (●). 5 mg per ml yeast alcohol dehydrogenase and 2×10^{-3} M 1,10-phenanthroline at time of 50% inhibition of catalytic activity, (■). Rotation determined in 1 mm path length cells using an AH-6 mercury lamp as the light source. (See Ref. 33.)

structural organization of the protein, allowing the empirical structural notation employed in this discussion. In the formulation $[(YADH)_4Zn_4](DPN)_4$, YADH represents a single unit of molecular weight 36,000 and $(YADH)_4$ the tetramer of molecular weight 151,000. The zinc atoms may here be viewed as bridges between four identical monomers resulting in the critical conformation of the tetramer, essential for activity, which is destroyed when the zinc bridges are abolished by the chelating agent. In conformity with both the inhibition kinetics and optical rotation, these structural changes, induced by chelating agents, are prevented competitively by DPN.

The observations gain significance both from the point of view of the study of the composition and configuration of the active site of the enzyme and of the chemical events of catalysis. Since OP competes with DPN for the active zinc atom, these structural properties of the enzyme can now be taken into consideration in delineating both presumable binding sites and the mode of binding of the coenzyme, leading to the development of reaction mechanisms which are internally more consistent than those which could be devised prior to the availability of this information. The requirements of stoichiometry and structure would suggest that DPN may act in conjunction with each of the four zinc atoms and also with each of the two adjacent subunits. The required topology could be accomplished readily were the coenzyme to cover the zinc-bridged gap between two subunits and interact at three points: a single site on each of the two adjoining peptide monomers and the third—weakly—with zinc (16). The demolition of the zinc bridge, irreversible thus far in this enzyme, and the consequent destruction of the three-point interaction would be manifested by the irreversible abolition of enzymatic activity.

The role of zinc in preserving the structural integrity has not been detected in liver alcohol dehydrogenase where the time-dependent, irreversible inhibition with 1,10-phenanthroline is not observed. Zinc is not removed and the molecular size does not change on exposure to this agent.

It has been emphasized previously that stability constants of metal chelates in aqueous solutions serve as inadequate guides for the prediction of the inhibition of this and other metalloenzymes (41). Steric hindrance, due to the quartenary structure of yeast alcohol dehydrogenase, may well be reflected in the modification of the dissociation constant of OP with the enzyme-bound zinc, higher by 5×10^{-3} than that with Zn^{++} ions in aqueous solution. The failure of Versene either to inhibit or dissociate the enzyme may be a specific example of steric hindrance.

These data do not answer directly the crucial question initially posed, i.e., precisely how the zinc atom is bound to the enzyme and how the interaction might result in catalytic activity. However, together with that already on record (29) the present information may illuminate at least the problem of the binding site. Zinc shows a marked preference for sulfur or nitrogenous ligand groups in the formation of its complexes. The striking stability of these complexes has been emphasized (54). It was suggested soon after the discovery of the metalloenzyme nature of yeast alcohol dehydrogenase that zinc may be bound as a mercaptide (41). The time-dependent displacement of zinc by Ag^+ ions and

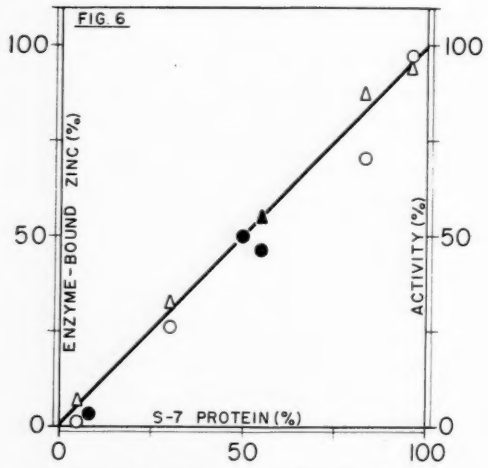


FIG. 6. Correlation between the effects of 1,10-phenanthroline and 8-hydroxy-quinoline-5-sulfonic acid on the activity, enzyme-bound zinc and structure of yeast alcohol dehydrogenase as determined in the ultracentrifuge. All measurements are expressed in per cent of the control in 0.1 M sodium phosphate buffer. The enzyme, 5×10^{-5} M, in 0.1 M sodium phosphate, pH 7.5, 0°, was incubated with 7.5×10^{-3} M 1,10-phenanthroline or 2×10^{-2} M 8-hydroxyquinoline-5-sulfonic acid. At various times aliquots were analyzed for activity and protein-bound zinc and examined in the ultracentrifuge. The percentage of the original enzyme remaining (S-7) is plotted on the abscissa, enzymatic activity after incubation with 1,10-phenanthroline (○) or with 8-hydroxyquinoline-5 sulfonic acid (●) on one ordinate and protein-bound zinc after incubation with 1,10-phenanthroline (△) or with 8-hydroxyquinoline-5-sulfonic acid (▲) on the other. (See Ref. 16.)

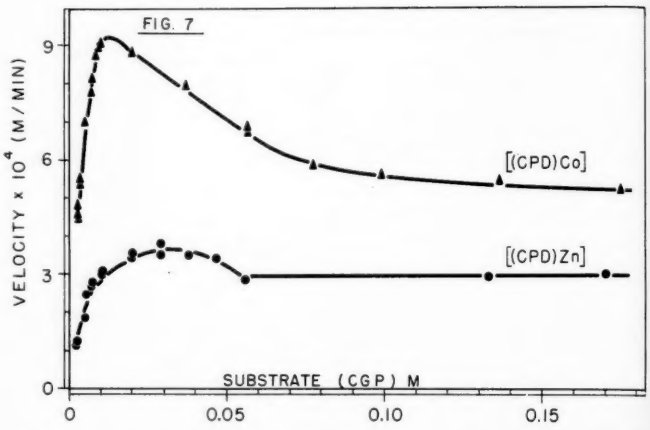


FIG. 7. Activities of zinc and cobalt carboxypeptidase as a function of substrate concentration. Zinc carboxypeptidase (●), cobalt carboxypeptidase (▲). Enzyme, 4.5×10^{-8} M in 0.02 M Na veronal, 1 M NaCl, pH 7.5, 25° was assayed varying the initial substrate (CGP) concentrations. The cobalt enzyme is more active over the entire range of substrate concentrations tested.

p-chloromercuribenzoate, which results in the formation of smaller fragments, supports this contention (29). These findings cannot be employed as unequivocal evidence for the existence of zinc mercaptide bonds maintaining the tetrameric structure, but they are consistent with such a hypothesis.

Addition of zinc ions has thus far failed to bring about the polymerization of subunits with consequent reactivation. Since studies of rotatory dispersion do not indicate that the dissociation of yeast alcohol dehydrogenase by 1,10-phenanthroline is accompanied by changes in the helical conformation of the protein, this irreversibility of the inactivation may be attributable to a *localized* configurational or chemical change at the zinc binding site. If zinc is bound to mercapto groups, their oxidation subsequent to the removal of zinc could readily account for this failure to observe reactivation.

CARBOXYPEPTIDASE OF BOVINE PANCREAS[4]

The size, the number of zinc atoms and coenzyme molecules, its high degree of lability and the involved inhibition kinetics, all complicate the exploration of the mode of action of the yeast alcohol dehydrogenase molecule and the delineation of an active center.

In contrast, many features of the carboxypeptidase molecule make it attractive as a model system, both to explore the characteristics of its active catalytic site and to study the mode of action of metals in enzymatic catalysis. In 1954, Vallee and Neurath demonstrated pancreatic carboxypeptidase to contain one gram atom of zinc per molecular weight of 34,300 (43, 44). The specificity of association between zinc and protein in the native enzyme has been corroborated further by the isolation and crystallization of a biosynthetically labeled, radioactive enzyme [(CPD)Zn^{65}], obtained from bovine and canine pancreatic secretions collected from live animals (22, 25). Bovine procarboxypeptidase A, the precursor, contains significant amounts of zinc, iron and nickel (21, 45). A preliminary report indicates that carboxypeptidase B, like carboxypeptidase A, is a zinc enzyme (10).

Carboxypeptidase is inhibited by complexing agents: 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, α,α'dipyridyl, KCN, Versene, zincon and sodium diethyldithiocarbamate (44), NaCN, Na_2S, sodium citrate, sodium oxalate and cysteine (28). The inhibition of some of these agents can be prevented by the formation of their Fe^{++}, Cu^{++} or Zn^{++} complexes (34).

While a stoichiometric amount of zinc is bound to native carboxypeptidase with sufficient firmness so that it accompanies the protein through isolation and successive crystallizations, it can be removed by dialysis either against buffers at low pH or 1,10-phenanthroline. Regardless of the means of removal, the loss of enzymatic activity is always directly proportional to the loss of metal (46, 47).

[4] In the following carboxypeptidase refers to carboxypeptidase A only, unless specified otherwise. R. J. P. Williams has recently concluded that a zinc-sulfur bond exists in carboxypeptidase A (personal communication; *Nature, London,* in press).

Within the limits of measurement, removal of zinc is complete at pH 3.4 after approximately 10 hours. The resultant crystalline metal-free apocarboxypeptidase is homogeneous by physical-chemical criteria, stable, and enzymatically inactive (*vide infra*). However, enzymatic activity may be regained by the addition of zinc to the apoenzyme. Again, reactivation is directly proportional to the amount of zinc bound by the enzyme, up to one gram atom per mole of protein (47). The reconstituted enzyme is enzymatically, chemically and crystallographically indistinguishable from native carboxypeptidase (2).

It is of considerable biological and chemical significance that peptidase activity toward carbobenzoxyglycyl-L-phenylalanine (CGP) expressed as the proteolytic coefficient, (C), can be restored to the metal free protein not only by zinc, but also by certain ions of the first transition period, in the order $Co^{++} > Ni^{++} > Zn^{++} > Mn^{++} > Fe^{++}$. Under the conditions described (3) the cobalt enzyme demonstrates 180–220 per cent, and the nickel enzyme from 110–130 per cent of the activity of the zinc enzyme.

The activities of the zinc and cobalt enzymes have been compared over a wide range of concentration of substrate. With carbobenzoxyglycyl-L-phenylalanine as substrate, under the conditions indicated in Figure 7, the activity of the cobalt enzyme is consistently higher than that of the zinc enzyme, though the ratio varies from a minimum of about 200 to a maximum of 400 per cent (1). Between pH 6 and 10 the cobalt and nickel carboxypeptidases are much more sensitive to pH than the zinc enzyme (3). Moreover, various metallocarboxypeptidases exhibit differential activity toward several peptide substrates, indicating that the specificity of the apoenzyme varies as a function of the metal (Table 1). It is worthy of note, therefore, that the same order of effectiveness of different metallocarboxypeptidases described for the hydrolysis of CGP does not prevail when carbobenzoxyglycyl-L-tryptophan or benzoylglycyl-L-phenylalanine are the substrates. With both of these substrates the zinc enzyme is more active than the cobalt and nickel enzymes, while the activity of the manganese enzyme is the same with all of these. None of these metalloenzymes hydrolyze carbobenzoxyglycylglycine. It is most remarkable that the rates at which peptide substrates are hydrolyzed by carboxypeptidase can be affected markedly and differentially by the particular metal which occupies the active site (3a, 4, 5).

TABLE 1. *Peptidase and Esterase Activities of the Metallocarboxypeptidases*

Metallocarboxypeptidase	Peptidase Activity† (C)				Esterase‡ Activity ($k \times 10^{-3}$)
[(CPD)Me]*	CGP	CGT	CGG	BGP	HPLA
[(CPD)Zn]	7.5	2.8	0	5.5	1.15
[(CPD)Co]	12.0	2.7	0	5.2	1.10
[(CPD)Ni]	8.0	2.0	0	2.0	1.00
[(CPD)Mn]	0.6	0.6	0	0.6	0.40
[(CPD)Hg]	0	0	0	0	1.34
[(CPD)Cd]	0	0	0	0	1.75

CGP, carbobenzoxyglycyl-L-phenylalanine; CGT, carbobenzoxyglycyl-L-tryptophan; CGG, carbobenzoxyglycylglycine; BGP, benzoylglycyl-L-phenylalanine; HPLA, hippuryl-dL-β-phenyl lactate.

* 1 gm atom of metal per mole of protein. † Assays were carried out at 0° using 0.02 M substrate and are expressed as the proteolytic coefficient, (C) (3). ‡ Assays were carried out at 25° using 0.01 M substrate and are expressed as the zero order velocity constant, k (30).

This is apparent in still another manner. Zinc carboxypeptidase and metallocarboxypeptidases containing other metals of the 1st transition group of the periodic table exhibit activity toward ester substrates (30). In regard to this activity toward ester substrates, such as hippuryl-dL-phenyl lactate (HPLA), some interesting consequences of metal replacement are observed. The carboxypeptidases containing the group IIB elements cadmium and mercury do not hydrolyze peptide substrates but exhibit esterase activity (3a, 4). The activities toward both CGP and HPLA of a number of metallocarboxypeptidases are also shown in Table 1.

The formation of the cobalt enzyme is accompanied by the appearance of an absorption maximum at 530 mμ, attributable to the cobalt ion, imparting a distinctive, reddish color to the crystalline enzyme, absent in the zinc enzyme (3) and suggesting binding to sulfur (54a).

The zinc and cobalt metallocarboxypeptidases are interconvertible: dialysis of the cobalt enzyme against a solution of zinc ions results in complete replacement of cobalt. The resultant decrease in enzymatic activity as a function of time is exactly as predicted on the basis of the mole fractions of the cobalt and zinc enzymes present at any one time. The reverse reaction, the displacement of zinc by cobalt, requires dialysis against solutions containing a 100-fold molar excess of cobalt for longer periods of time. The increase in enzymatic activity is exactly as predicted from the metal composition of the mixture (3).

It is clear, therefore, that zinc and cobalt are bound to the same site on the protein. At the pH of their maximal activity one gram atom per mole of each of these or of nickel and manganese is bound to the apoenzyme.

Whereas zinc remains firmly bound and activity is unchanged during 6 days of dialysis against metal-free buffer at neutral pH, under the same conditions the cobalt enzyme loses 40 per cent of the bound metal and *pari passu*, its activity (3). The apparent dissociation constants of a number of metallocarboxypeptidases have been estimated from equilibrium dialysis measurements in tris(hydroxymethyl)aminomethane buffer at pH 8.0 as shown in Table 2, Cols. 1 and 2. The apparent stability constant is 2.1×10^8 for the zinc enzyme, higher than any other among the metallocarboxypeptidases measured thus far, though the other group IIB elements are next highest (4). Allowing for the tris and chloride, the latter required to dissolve the enzyme, an appropriate correction must be made for each to ascertain the true stability

TABLE 2. *Stability Constants of the Metallocarboxypeptidases (4, 50a)*

Metallocarboxypeptidase [(CPD)Me]*	1 Stability Constant (app.†)	2 Log Stability Constant (app.)	3 Log Stability Constant (corr.)
[(CPD)Mn]	4.0×10^5	5.6	5.6
[(CPD)Co]	6.7×10^5	5.8	7.0
[(CPD)Ni]	5.0×10^5	5.7	8.2
[(CPD)Cu]	1.4×10^6	5.1	10.6
[(CPD)Zn]	2.1×10^8	8.3	10.5
[(CPD)Cd]	8.4×10^7	7.9	10.8
[(CPD)Hg]	5.0×10^6	6.7	21.0

* 1 gm. atom of metal per mole of protein. † Determined in 1 M sodium chloride, 0.05 M tris, pH 8.0, 4°C. ‡ Corrected for the reduction of (Me_{free}) by the competing tris and Cl^- complexes.

constants. These are shown in Table 2, column 3. The corrected constants are in the order $Hg^{++} > \gg Cd^{++} > Zn^{++} > Ni^{++} > Co^{++} > Mn^{++}$. These facts reflect on the presumable chemical nature of the binding sites on the protein[5] (*vide infra*).

However, such data do not, of course, yield information concerning the binding of the substrate. The faculty jointly to remove and restore zinc and activity permits an experimental examination of substrate binding. Insight into the mode of binding of the substrate to the enzyme may be gained by means of the classical procedures of inhibition kinetics as discussed previously in regard to alcohol dehydrogenase (14, 55). As has been pointed out, the use of chelating agents in such studies can only define the role which the *metal atom* may play in substrate binding but cannot identify the existence of additional, organic binding sites. Varying the order of addition of metal and substrate to the metal-free enzyme allows a different approach to the problem as shown in Figure 8. When Zn^{++} ions are added to the apoenzyme, (CPD), followed by substrate, the initial rates of the reconstituted enzyme are identical with those of the native enzyme, [(CPD)Zn]. When substrate is added first and is then followed by Zn^{++} ions, compared to the zinc-free control there is virtually no restoration of activity as is apparent from the measurement of initial rates (9). This and similar experiments demonstrate that the presence of the substrate prevents the otherwise instantaneous, complete reconstitution of the active metalloenzyme on mixing equimolar quantities of the apoenzyme with Zn^{++} ions. Since the substrate itself binds Zn^{++} ions only weakly, it appears reasonable to interpret this phenomenon as evidence for the presence of substrate binding groups on the apoenzyme. Thus, when a substrate molecule attaches to the apoenzyme it may be thought to cover the specific site of binding for the zinc atom, preventing its reentry. Since kinetic studies indicate that the metal also participates in the binding of substrate (9), a three-point substrate attachment may again be postulated.

[5] The pertinence of these constants to the identification of the metal binding site has been discussed elsewhere (3a, 4, 50a).

Electrophoretic and sedimentation analyses and measurements of optical rotation and x-ray diffraction of the inactive, zinc-free apocarboxypeptidase failed to demonstrate any significant differences in comparison to the native enzyme, though a greater susceptibility to denaturation was apparent (26). There are indications, however, that there may be structural changes, limited to a discrete region surrounding the metal atom. Equilibrium dialysis indicates (26) that the metal-free protein fails to bind phenylacetate, an inhibitory, structural analog of a specific substrate of the enzyme (8). This all-or-none difference in behavior toward phenylacetate demonstrates that in native carboxypeptidase the metal either coordinates with the inhibitor, or confers a configuration on the enzyme which is essential for the formation of the protein-inhibitor complex (22). These studies provide an independent verification of the suggestions advanced on the basis of the experiments involving the order of addition of Zn^{++} ions and substrate to (CPD).

However, such studies do not resolve the question of precisely where the metal is bound to the protein or in what manner this combination results in catalytic activity. Our recent investigations have yielded important experimental leads through which to answer these questions.

We have established that the activity of carboxypeptidase depends crucially on the binding of the one zinc atom to one mercapto group of the metal-free, inactive apocarboxypeptidase (39). The native and reconstituted zinc enzymes do not react with silver, *p*-chloromercuribenzoate or ferricyanide under widely varying conditions of pH, buffers, ionic strength, temperature and time of exposure to these agents, indicating the absence of titratable —SH groups. In tris buffer the active zinc-enzyme is not inhibited by these agents.

The inhibition of the enzyme with iodoacetate, cuprous and lead ions in veronal buffer had led to the suggestion that sulfhydryl groups might be essential for activity. However, the lack of inhibition with silver and mercuric ions, which generally act as sulfhydryl inhibitors, had to be attributed to unknown factors (28). The presence of the zinc mercaptide can now explain this apparent discrepancy.

In contrast, the zinc-free, inactive apocarboxypeptidase interacts with approximately one mole of Ag^+, *p*-chloromercuribenzoate or ferricyanide, disclosing a reactive —SH group. Additions of mole fractions of zinc to the zinc-free apoenzyme results in a commensurate decrease in the mole fraction of —SH titratable with Ag^+, documenting the concealment of this reactive sulfur atom by zinc (Fig. 9). The sum of the number of gram atoms of zinc bound to the enzyme and of the number of —SH titrated per mole of enzyme equals 1. *p*-Chloromercuribenzoate, highly specific for the—SH group, also blocks its titration with Ag^+ ions (Fig. 9).

This concealed mercapto group has a crucial functional role through its binding with zinc. Preincubation of the metal-free enzyme with an excess of silver or

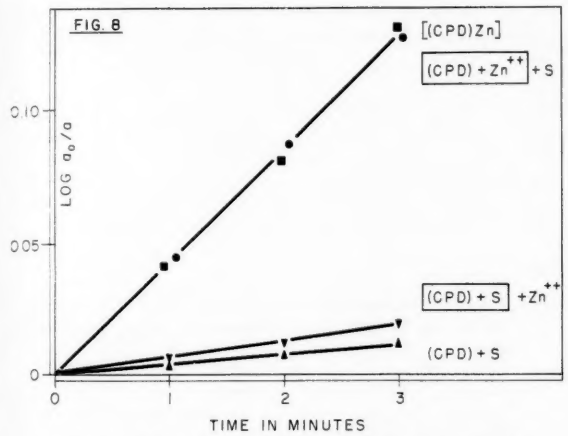
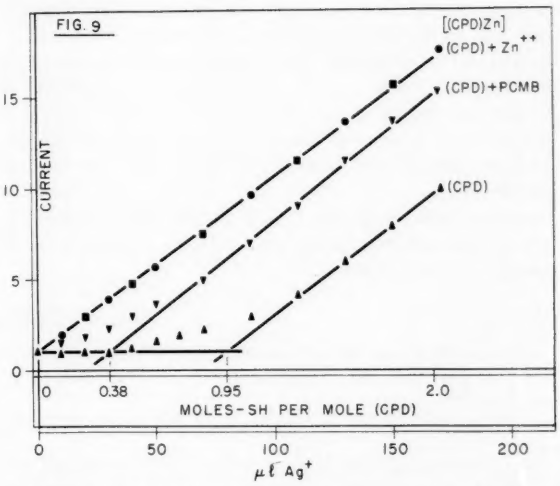


FIG. 8. Prevention of restoration of activity to apocarboxypeptidase, (CPD), by Zn^{++} by substrate, S. Native zinc carboxypeptidase (■), apocarboxypeptidase + CGP (▲), 1 gram atom Zn^{++} + apocarboxypeptidase were first incubated for 1 minute and then CGP added to start the reaction (●); CGP + apocarboxypeptidase were first incubated for 1 minute and then Zn^{++} added to start the reaction (▼). Assay conditions, enzyme 1 × 10^{-6} M, substrate is 0.02 M CGP, 0.1 M tris, 1 M NaCl, pH 7.5, 0°. The initial first order rates were measured as in (3). Preincubation



of (CPD) with the substrate CGP prevents restoration of activity with Zn^{++}.

FIG. 9. Demonstration of a mercapto group in apocarboxypeptidase, (CPD), its concealment in the presence of zinc and its blockage by *p*-chloromercuribenzoate in the absence of zinc. Native zinc carboxypeptidase (■), apocarboxypeptidase (▲), apocarboxypeptidase + 1 gram atom Zn^{++} (●), apocarboxypeptidase + 2 moles of PCMB, (▼). Ag^{+} -tris amperometric titrations, pH 7.5, 4° as in (39).

p-chloromercuribenzoate blocks the *restoration* of activity to the metal-free enzyme by subsequent additions of stoichiometric quantities of zinc ions, which restore full activity to the untreated apocarboxypeptidase.

Cysteine is the most probable amino acid residue forming the zinc mercaptide which is essential for activity. The fact that this one zinc atom is bound to the only mercapto group identifiable in carboxypeptidase limits the localization of the zinc mercaptide involved in activity to one particular residue in the amino acid sequence of the enzyme. It was suggested that zinc is additionally bound to a nitrogen atom (39).

SUMMARY

It is thus clear from the evidence available that the role of zinc in alcohol dehydrogenase of yeast must include its participation in maintaining protein structure while being involved in activity. The available data suggest that the metal is bound as a dimercaptide between two adjacent subunits of the molecule while it maintains the molecular configuration which is crucial to the binding of DPN by a three-point attachment: one each of two adjacent subunits and the third to zinc. The marked Cotton effect establishes the existence of a protein-oriented, stereo-specific zinc phenanthroline chromophore at an active zinc site of this enzyme, rationalizing the stereospecificity of the hydrogen transfer to DPN (51a). Thus, the fundamental features essential to the maintenance of the active site of this enzyme are beginning to emerge. The identification of a zinc mercaptide as a component of the active center of carboxypeptidase seems directly pertinent to the definition of the "active sites" of other zinc enzymes. Such characterization of the molecular details of an active site becomes feasible in an intact enzyme through features peculiar to metalloenzymes.

CONCLUSION

In an excellent treatise on the functional importance of chemically identifiable and biologically active groups of enzymes, Fraenkel-Conrat points out that the use of metals as specific reagents for the study of active catalytic sites represents a field that has merely been suggested and has not as yet been explored (12). The metal in metalloenzymes may be regarded as a reagent so specific for the active binding site of the enzyme that the biological system itself employs it as a marker of the active site. It is not surprising, of course, that the interaction of the same metal, e.g., zinc, with different apoenzymes, i.e., apoalcoholdehydrogenase and apocarboxypeptidase, will result in totally different catalytic properties. One would expect the individual enzyme protein to make exactly such a contribution to specificity. The present views relegate specificity to an area of the protein which is relatively small, as compared with the total size of the molecule. In metalloenzymes the composition and conformation of the binding site in conjunction with the metal atom thus determine catalytic specificity.

Differences in the molecular organization of the active sites of different zinc enzymes such as alcohol dehydrogenase and carboxypeptidase must be subtle. A three-point attachment of coenzyme or substrate to metal and apoenzyme postulated in both instances, no doubt, constitutes one means of achieving specificity over and

beyond that of the metal atom itself which is also directly involved in the catalytic process. The discernment of the details of this molecular organization of metalloenzymes is susceptible to an approach utilizing an integration of analytical, organic, and physical-chemical principles to discern the chemical basis of biological specificity. Importantly, in metalloenzymes the unique association of biological activity with the metal atom allows a chemical differentiation of the active center from the remainder of the molecule, thus offering unusual experimental opportunities.

It may well be that the study of these particular systems may provide an important basis for the detection of chemical properties and mechanisms which serve to achieve the exquisite specificity which is characteristic of biological systems in general.

The original work here reported would never have been accomplished without the diligence and enthusiasm of all my associates. It is with special pleasure that I acknowledge the contributions of my collaborators, at long distance, Professor H. Neurath, Department of Biochemistry, University of Washington, Seattle and Dr. R. J. P. Williams, Wadham College, Oxford University.

REFERENCES

1. ALLAN, B. J. AND B. L. VALLEE. In preparation.
2. COLEMAN, J. E., B. J. ALLAN AND B. L. VALLEE. *Science* 131: 350, 1960.
3. COLEMAN, J. E. AND B. L. VALLEE. *J. Biol. Chem.* 235: 390, 1960.

3a. COLEMAN, J. E. AND B. L. VALLEE. *Federation Proc.* 20: 220, 1961.

4. COLEMAN, J. E. AND B. L. VALLEE. *J. Biol. Chem.* In press.
5. COOMBS, T. L. AND B. L. VALLEE. In preparation.
6. DJERASSI, C. *Optical Rotatory Dispersion.* New York: McGraw Hill, 1960.
7. EHRENBERG, A. *Acta Chem. Scand.* 11: 1257, 1957.
8. ELKINS-KAUFMANN, E. AND H. NEURATH. *J. Biol. Chem.* 178: 645, 1949.
9. FELBER, J. P., T. L. COOMBS AND B. L. VALLEE. In preparation.
10. FOLK, J. E., J. A. GLADNER, W. R. CARROLL AND K. A. PIEZ. *Fed. Proc.* 19: 216, 1960.
11. FRAENKEL-CONRAT, H. In: *Methods in Enzymology*, vol. 4, edited by S. P. Colowick AND O. N. Kaplan. New York: Acad. Press, 1957, p. 247.
12. FRAENKEL-CONRAT, H. In: *The Enzymes* (2nd Ed.), vol. I, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 589.
13. HOCH, F. L. AND B. L. VALLEE. *J. Biol. Chem.* 221: 491, 1956.
14. HOCH, F. L., R. J. P. WILLIAMS AND B. L. VALLEE. *J. Biol. Chem.* 232: 453, 1958.
15. KÄGI, J. H. R. *Fed. Proc.* 18: 484, 1959.
16. KÄGI, J. H. R. AND B. L. VALLEE. *J. Biol. Chem.* 235: 3188, 1960.
17. KEILIN, D. AND T. MANN. *Biochem. J.* 34: 1163, 1940.
18. LEHNINGER, A. L. *Physiol. Rev.* 30: 393, 1950.
19. LINDERSTROM-LANG, K. U. AND J. A. SCHELLMAN. In: *The Enzymes* (2nd Ed.), vol. I, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, p. 433.
20. MATHIES, J. *J. Biol. Chem.* 233: 1121, 1958.
21. NEURATH, H. *Discussions Faraday Soc.* 29: 257, 1955.
22. NEURATH, H., J. A. RUPLEY AND B. L. VALLEE. In: *Amino Acids, Proteins and Cancer Biochemistry*, edited by J. T. EDSALL. New York: Acad. Press, 1960, p. 43.
23. PLOCKE, D. J., D. LEVINTHAL AND B. L. VALLEE. *Meetings of the American Chemical Society*, New York, September, 1960.
24. REDETZKI, H. E. AND W. W. NOWINSKI. *Nature, London* 179: 1018, 1957.
25. RUPLEY, J. A. AND H. NEURATH. In preparation.
26. RUPLEY, J. A. AND H. NEURATH. *J. Biol. Chem.* 235: 609, 1960.
27. SCHELLMAN, C. G. AND J. A. SCHELLMAN. *Compt. rend. trav. lab. Carlsberg, Ser. chim.* 30: 463, 1958.
28. SMITH, E. L. AND H. T. HANSON. *J. Biol. Chem.* 179: 803, 1949.
29. SNODGRASS, P. J., B. L. VALLEE AND F. L. HOCH. *J. Biol. Chem.* 235: 504, 1960.
30. SNOKE, J. E., G. W. SCHWERT AND H. NEURATH. *J. Biol. Chem.* 175: 7, 1948.
31. THEORELL, H., A. P. NYGAARD AND R. K. BONNICHSEN. *Acta Chem. Scand.* 9: 1148, 1955.
32. ULMER, D. D. AND B. L. VALLEE. *Fed. Proc.* 19: 27, 1960.
33. ULMER, D. D. AND B. L. VALLEE. *J. Biol. Chem.* 236: 730, 1961.
34. VALLEE, B. L. *Advances Protein Chem.* 10: 317, 1955.
35. VALLEE, B. L. In: *Fourth International Congress of Biochemistry*, Vol. III, Proteins, edited by H. Neurath and H. Tuppy. New York: Pergamon, 1960, p. 138.
36. VALLEE, B. L. In: *The Enzymes* (2nd Ed.), vol. III, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1960, p. 225.
37. VALLEE, B. L., S. J. ADELSTEIN AND J. A. OLSON. *J. Am. Chem. Soc.* 77: 5196, 1955.
38. VALLEE, B. L. AND T. L. COOMBS. *J. Biol. Chem.* 234: 2615, 1959.
39. VALLEE, B. L., T. L. COOMBS AND F. L. HOCH. *J. Biol. Chem.* 235: PC 45, 1960.
40. VALLEE, B. L., T. L. COOMBS AND R. J. P. WILLIAMS. *J. Am. Chem. Soc.* 80: 397, 1958.
41. VALLEE, B. L. AND F. L. HOCH. *Proc. Nat. Acad. Sc., U. S.* 41: 327, 1955.
42. VALLEE, B. L. AND F. L. HOCH. *J. Biol. Chem.* 225: 185, 1957.
43. VALLEE, B. L. AND H. NEURATH. *J. Am. Chem. Soc.* 76: 5006, 1954.
44. VALLEE, B. L. AND H. NEURATH. *J. Biol. Chem.* 217: 253, 1955.
45. VALLEE, B. L., H. NEURATH AND P. J. KELLER. Unpublished observations.
46. VALLEE, B. L., J. A. RUPLEY, T. L. COOMBS AND H. NEURATH. *J. Am. Chem. Soc.* 80: 4750, 1958.
47. VALLEE, B. L., J. A. RUPLEY, T. L. COOMBS AND H. NEURATH. *J. Biol. Chem.* 235: 64, 1960.
48. VALLEE, B. L., E. A. STEIN, W. A. SUMMERWELL AND E. H. FISCHER. *J. Biol. Chem.* 234: 2901, 1959.
49. VALLEE, B. L. AND W. E. C. WACKER. *J. Biol. Chem.* 78: 1771, 1956.
50. VALLEE, B. L., R. J. P. WILLIAMS AND F. L. HOCH. *J. Biol. Chem.* 234: 2621, 1959.

50a. VALLEE, B. L., R. J. P. WILLIAMS and J. E. COLEMAN. *Nature: London* 190: 633, 1961.

51. WARBURG, O. *Heavy Metal Prosthetic Groups and Enzyme Action.* Oxford: Clarendon Press, 1949.

51a. WESTHEIMER, F. H., H. F. FISHER, E. E. CONN and B. VENNESLAND. *J. Am. Chem. Soc.* 73: 2403, 1951.

52. WILKINS, R. G. AND M. J. G. WILLIAMS. In: *Modern Coordination Chemistry* edited by J. Lewis and R. G. Wilkins. New York: Interscience, 1960, p. 174.
53. WILLIAMS, R. J. P. *Biol. Rev.* 28: 4, 1953.
54. WILLIAMS, R. J. P. In: *The Enzymes* (2nd Ed.), edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, vol. I, p. 391.

54a. WILLIAMS, R. J. P. *Nature: London* 88: 322, 1960.

55. WILLIAMS, R. J. P., F. L. HOCH AND B. L. VALLEE. *J. Biol. Chem.* 232: 465, 1958.
56. WILLSTATTER, R. *J. Chem. Soc.* 1359, 1927.

DISCUSSION

Metalloenzymes in the study of active enzyme sites

ESMOND E. SNELL

Department of Biochemistry, University of California, Berkeley, California

I SHOULD LIKE TO CONGRATULATE Dr. Vallee on his lucid description of the exemplary experiments conducted by his group that demonstrate Zn^{++} as one functional active site in the several enzymes he has discussed. It should be emphasized that only through careful studies of this nature, conducted with highly purified enzymes, will we gain a true understanding of the nature of the collaborative catalytic role played by metal and protein in the metalloenzymes and develop a rational basis for the application of chelating agents in medicine.

In my brief comments, today, I should like to emphasize three points that have not been specifically recognized in our discussions so far. These are: *a*) the utility of study of model systems involving metal ions in clarifying the nature of the "active site" of enzymes and, also, in recognizing possible roles played by portions of the protein chain that are not a part of the immediate "active site"; *b*) the variability in the metal ion requirement for individual enzymatic functions *in vivo*; and *c*) the corresponding variability and latitude exhibited in metal ion requirements of living organisms.

A. Model systems involving metal ions. Point *a* can be illustrated by reference to our studies [recently reviewed (16), cf. also (11)] on catalysis of reactions of amino acids by pyridoxal and metal ions. Briefly we have shown that in the presence of pyridoxal (or pyridoxal phosphate) and an appropriate metal ion (e.g. Cu^{++}, Al^{+3} or Fe^{+3}) amino acids undergo a whole series of reactions that are catalyzed in living organisms by pyridoxal phosphate proteins. Examples are listed in Table 1. In the absence of metal ions and with added EDTA, such nonenzymatic model reactions (decarboxylation excepted) proceed at only 5 to 10 per cent of the rate achieved in the presence of the metal ions. Catalysis of the reactions has been attributed to formation of a ternary complex involving metal ion, pyridoxal and amino acid in which the electron density about the α-carbon atom of the amino acid residue is greatly reduced and the chemical bonds correspondingly labilized so that one or more of the observed reactions of Table 1 may ensue, depending upon other environmental conditions (11, 16). Such transformations leading to transamination or to racemization of amino acids are shown in Figure 1.

The amazing correspondence between the nonenzymatic reactions of amino acids that occur in these chemical systems and the corresponding reactions catalyzed by naturally-occurring pyridoxal phosphate proteins leaves no room for doubt that pyridoxal phosphate is the "active site" of each of these enzymes. Availability of the metal ion-catalyzed model systems has permitted an assessment of the importance of each of the substituting groups in the pyridoxal molecule for catalysis of these reactions, so that the relationship of the chemical structure of this "active site" to its catalytic action is perhaps better understood than is that of any other naturally-occurring cofactor.

Only a very few pyridoxal phosphate enzymes have been obtained in reasonably pure form [e.g. glutamic aspartic transaminase (2–4) and homoserine dehydrase (8)], but these do not contain stoichiometric amounts of metal ions and are not dependent upon added ions for their activity. It is, therefore, clear that the role played by metal ions in catalysis of the nonenzymatic reactions is filled even more efficiently by protein in the pyridoxal phosphate enzymes. We have suggested elsewhere (16, 17) that this role may be manifold, consisting in part in facilitating formation of the complex between pyridoxal and amino acid, in part in stabilizing this intermediate in a form that can undergo the desired reactions, and in part in providing an electro-negative group in addition to that of pyridoxal itself that facilitates the electron displacements that are prerequisite to the group of reactions catalyzed by these systems. More detailed study of these systems undoubtedly would further clarify and delineate this role, and thus suggest ways in which the protein portion of the corresponding enzyme may fill its function in promoting these same reactions. These points have been considered in more detail elsewhere (16, 17).

B. Variability in metal ion requirements of individual enzymes. We have seen above how the role played by metal ions in the model reactions between pyridoxal and

TABLE 1. *Pyridoxal-Catalyzed Model and Enzymatic Reactions*

Transamination reactions:

$$\text{Keto acid}_1 + \text{amino acid}_2 \rightleftharpoons \text{keto acid}_2 + \text{amino acid}_1$$

Racemization:

$$\text{D-amino acids} \rightleftharpoons \text{L-amino acids}$$

Aldol reactions:

$$RCHOHCHNH_2COOH \rightleftharpoons RCHO + H_2NCH_2COOH$$

e.g. serine aldolase, threonine aldolase

α,β-Elimination reactions:

$$RCHXCHNH_2COOH + H_2O \rightarrow HX + NH_3 + RCH_2COCOOH$$

e.g. Dehydration of serine, threonine
Tryptophanase and cystathionase reactions
Desulfhydration of cysteine, etc.

Decarboxylation reactions: $RCHNH_2COOH \rightarrow CO_2 + RCH_2NH_2$

Synthetic reactions: e.g. tryptophan and cysteine synthesis

$$HOCH_2CHNH_2COOH + HX \rightarrow XCH_2CHNH_2COOH + H_2O$$

amino acids is played even more efficiently by protein in the corresponding enzymatic reactions. Do analogous cases occur in living organisms, where the function played by a metalloenzyme in one organism is independent of metal ions in another organism? Unfortunately, no extended study of this point has been made. It should be noted, however, that the crystalline aldolase of yeast *requires* Zn^{++}, Fe^{++} or Co^{++} for activity (22), whereas crystalline muscle aldolase contains no metal ions and requires none as an activator (20, 21). Similar differences in the enzymes concerned may explain the apparent obligatory requirement for Zn^{++} for action of purified alcohol dehydrogenase and several other DPN-dependent enzymes (1, 19), as opposed to the closely related lactic dehydrogenase, which appears to be independent of Zn^{++} in its action (18).

Much more common than differences of this type are those cases in which a given metal-activated enzyme may function (albeit at somewhat different rates) with any one of several different metal ions as activators. For example, a large number of enzymes is known that function interchangeably with Mg^{++} or Mn^{++} as the activating cation. A recent example from our own experience is provided by the reaction of equation 1, which is an essential reaction in those organisms that synthesize valine.

$$2\ \text{Pyruvate} \xrightarrow[Mg^{++}\text{ or }Mn^{++}]{\text{TPP}} \text{Acetolactate} + CO_2 \qquad (1)$$

The purified enzyme from *Neurospora crassa* functions equally well with either added Mn^{++} or added Mg^{++}, but the former is optimally active at about 0.1 the concentration of the latter. Ca^{++}, Fe^{+3} and Co^{++} show somewhat less activity (13). The corresponding enzyme from *Escherichia coli* shows a similar relationship to metal ions that differs only in detail (13). That limited interchangeability of the metal component of metalloenzymes which contain a firmly bound metal ion may also occur is shown by the elegant experiments just described by

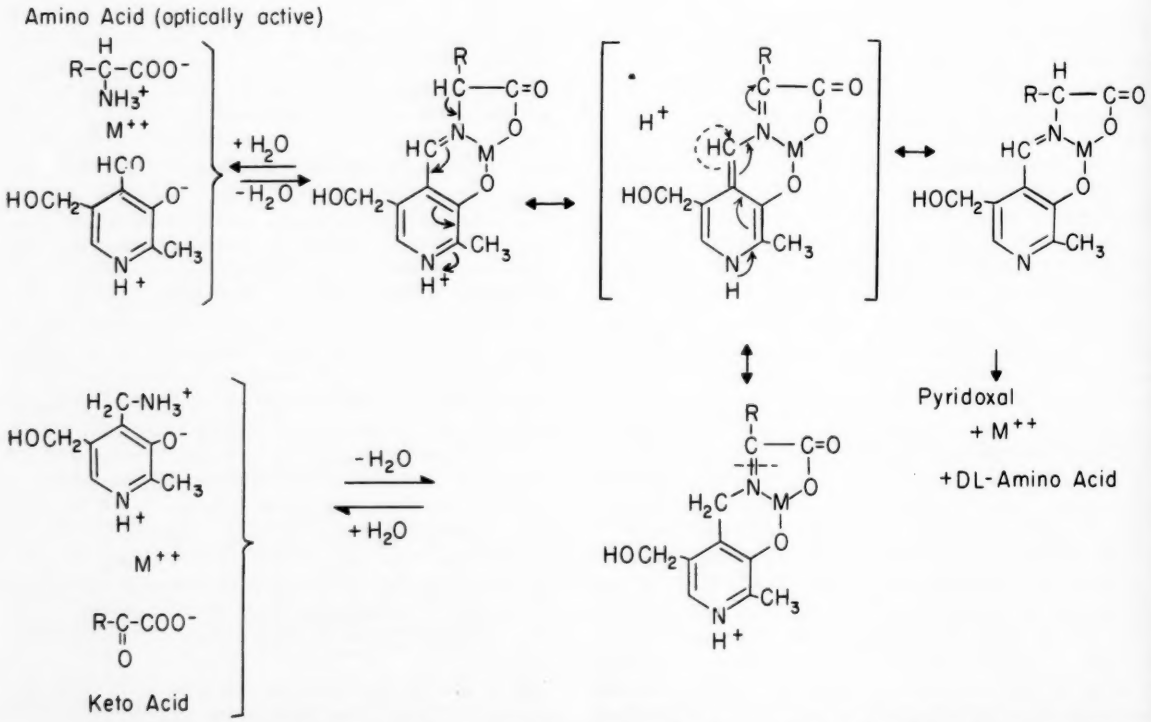


FIG. 1. Schematic diagram illustrating postulated transitional forms in the catalysis by pyridoxal and metal ions of transamination and racemization reactions of amino acids.

Dr. Vallee, in which the Zn^{++} ion of carboxypeptidase was replaced by any of several other ions to yield a functional peptidase. By way of contrast, the reductoisomerase from *N. crassa* and from *E. coli* that catalyzes a subsequent step in the synthesis of valine (equation *2*) is absolutely specific in its requirement

$$\text{Acetolactate} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{Mg}^{++}} \text{TPN}^+ + \underset{\alpha,\beta\text{-dihydroxyisovalerate}}{} \quad (2)$$

for Mg^{++}; no other metal ion can be utilized in its stead (14). Thus, there appear to exist in living tissues metabolic reactions which are specifically dependent on the presence of a single metal ion, reactions which occur if any one of several metal ions is supplied, and reactions which are metal ion dependent in one organism but not in another.

A final point relates to those enzymatic processes which can function with any one of several different metal activators. Here it can be observed that the most effective metal ion for the enzyme from one organism may not be the most effective for the corresponding enzyme from another organism. We have compared properties of the enzyme *pyridoxal kinase*, which catalyzes phosphorylation of pyridoxal according to equation *3*, from several sources.

$$\text{Pyridoxal} + \text{ATP} \xrightarrow[\text{or } \text{Mg}^{++}]{\text{Zn}^{++}} \text{ADP} + \text{pyridoxal phosphate} \quad (3)$$

The enzyme from human brain (10) and several other mammalian sources (9) is much more active with Zn^{++} than with Mg^{++} or Mn^{++} as the activating cation; the corresponding enzyme from *Lactobacillus casei* or *Streptococcus faecalis* on the other hand is much more active when supplied Mg^{++} in place of Zn^{++} (9).

C. Latitude in metal ion requirements of living organisms. In unicellular organisms, where the principal or exclusive role of essential metal ions is to serve as activators of various enzymes of the cell, one might expect the latitude that exists in the metal ion requirements of individual enzymes of the cell to be reflected in a corresponding latitude in the nutritional requirements of the cell for a given cation. Such observations have indeed been made. In *S. faecalis*, for example, the requirement for K^+ for growth can apparently be replaced by equimolar amounts of Rb^+ (5–7, 15). Similar findings have been made in *L. casei* (5–7) and in some varieties of *Chlorella* (12). For other organisms, such as a strain of *Streptococcus equinus* (5–7, 15) and some higher plants (12), rubidium, though not a complete replacement for K^+, greatly reduces the requirement for it. In a similar fashion, Sr^{++} replaces Ca^{++} in part or in whole for growth of certain organisms, and the requirement of many bacteria for Mn^{++} is greatly reduced by the presence in the medium of ample amounts of Mg^{++} (see (15) for references). Such findings were interpreted early [e.g. (5–7)] in terms of the hypothesis that many metal ion activated enzymes of the cell could be activated by more than a single ion, and that, to the extent that ion A replaced ion B for activation of a given reaction, the nutritional requirement for B could be decreased by providing an excess of A. In such cases, as pointed out elsewhere (5–7, 15) it is meaningless to inquire which is the "essential" ion; whichever functional ion that is available to the cell can be used. From considerations of this nature it becomes evident that living organisms may make more general use of the metal ions in their environment than is indicated by their minimum nutritional requirements. It was encouraging to learn that such interpretations now have an experimental basis not only for processes that require readily dissociable metal ion activators, but perhaps also for the true metalloenzymes in the sense of Dr. Vallee.

These remarks have indicated, I hope, not only the virtues of a detailed study of the catalytic activities of metal chelate systems in clarifying the nature and role of the active sites of certain enzymes, but also a few of the complexities that attach to the study of the role of metal ions in enzymatic and living systems. Adequate understanding of these complexities will require much more detailed study of purified systems similar to that we have heard described in this conference.

REFERENCES

1. Hoch, F. L. and B. L. Vallee. *J. Biol. Chem.* 221: 491, 1956.
2. Jenkins, W. T. and I. W. Sizer. *J. Biol. Chem.* 234: 1179, 1959.
3. Jenkins, W. T. and I. W. Sizer. *J. Biol. Chem.* 235: 620, 1960.
4. Jenkins, W. T., D. A. Yphantis and I. W. Sizer. *J. Biol. Chem.* 234: 51, 1959.
5. MacLeod, R. A. and E. E. Snell. *J. Biol. Chem.* 176: 39, 1948.
6. MacLeod, R. A. and E. E. Snell. *Ann. New York Acad. Sc.* 52: 1249, 1950.
7. MacLeod, R. A. and E. E. Snell. *J. Bact.* 59: 783, 1950.
8. Matsuo, Y. and D. M. Greenberg. *J. Biol. Chem.* 230: 561, 1958.
9. McCormick, D. B., M. Gregory and E. E. Snell. Manuscript in preparation.
10. McCormick, D. B. and E. E. Snell. *Proc. Nat. Acad. Sc.*, U. S. 45: 1371, 1959.
11. Metzler, D. E., M. Ikawa and E. E. Snell. *J. Am. Chem. Soc.* 76: 639, 1954.
12. Pirson, A. *Ann. Rev. Plant Physiol.* 6: 71, 1955.
13. Radhakrishnan, A. N. and E. E. Snell. *J. Biol. Chem.* 235: 2316, 1960.
14. Radhakrishnan, A. N. and E. E. Snell. *J. Biol. Chem.* 235: 2322, 1960.
15. Snell, E. E. In: *Trace Analysis*, edited by J. H. Roe and H. J. Koch. New York: Wiley, 1957, p. 547.
16. Snell, E. E. *Vitamins and Hormones* 16: 77, 1958.
17. Snell, E. E. and W. T. Jenkins. *J. Cell. & Comp. Physiol.*, Supp. 1 54: 161, 1959.
18. Terayama, H. and C. S. Vestling. *Biochim. et biophys. acta* 20: 586, 1956.
19. Vallee, B. L. *Advances Protein Chem.* 10: 317, 1955.
20. Warburg, O. *Schwermetalle als Wirkungsgruppen von Fermenten.* Berlin: Saenger, 1946, p. 164.
21. Warburg, O. and W. Christian. *Biochem. Ztschr.* 314: 149, 399, 1943.
22. Warburg, O., K. Gawehn and G. Lange. *Ztschr. Naturforsch.* 9b: 109, 1954.

DR. COTZIAS: Dr. Vallee, I would like to know if your alcohol dehydrogenases are sensitive to high concentrations of substrate and, if so, if this is a reversible or irreversible effect.

DR. VALLEE: At the substrate concentrations here employed neither ethanol nor acetaldehyde inhibit the enzyme.

DR. COTZIAS: How does this relate to the fact that you observe a diminution of this enzyme in cirrhosis of the liver?

DR. VALLEE: I am afraid I do not have any pertinent information.

DR. MARTIN: If the value of 10^5 for the formation constant for zinc ion binding at the sulfhydryl group of glutathione may be applied to proteins, your value of zinc ion binding to carboxypeptidase of about 10^8 indicates that additional interaction(s) occur. To what group(s) in addition to the sulfhydryl might the zinc ion be bound?

DR. VALLEE: All of the data point to a nitrogen atom as the second binding site. Your data on glutathione certainly are consistent with those presented here for carboxypeptidase.

DR. GUSTAFSON: Were your centrifugation experiments carried out in the presence of a supporting electrolyte or just as the zinc enzymes alone?

DR. VALLEE: They were performed in 0.1 M sodium phosphate buffer at pH 7.5.

DR. SCHWARZ: When visiting last year with Prof. Dr. G. Weitzel at the University of Tubingen, Germany we discussed the problem of trace element distribution in tissues, especially as it relates to diabetes. Dr. Weitzel has done much interesting work on zinc (see also Dr. Philips, these proceedings). He stressed that zinc is much more abundant than any other trace element in the tissues. There is even more zinc than iron, if one excludes the iron in erythrocytes. The amount of zinc far exceeds that which could be accounted for in terms of specific zinc enzymes. It seems conceivable that the zinc has a more nonspecific function, namely, that it acts as a masking or buffering agent to protect sensitive sites of enzymes. These may, at the moment or at the site of action, be taken up by the truly effective factors, such as other, more specific trace metals. This possible mode of action of zinc seems quite plausible, and it appears as if some of the results presented by Dr. Vallee actually may be used as proof for this hypothesis. If it is true that zinc acts as a nonspecific masking agent, we should be able to isolate a whole series of other zinc containing enzymes which are not *truly* zinc enzymes, so to speak. I would like to have Dr. Vallee's reaction to this thought.

DR. VALLEE: Actually I have pointed out repeatedly (Vallee, B. L. *J.A.M.A.* 162: 1053, 1956; Vallee, B. L. *Physiol. Rev.* 39: 443, 1959), that the adult human body contains two to three grams of zinc as contrasted to four to six grams of iron. Unfortunately, little attention has been given to this information. Much of the iron has been accounted for. Given some time, I believe that one will find the enzymes which contain zinc.

DR. SCHEINBERG: I wondered why you hadn't tried copper as a substitute for zinc in apocarboxypeptidase.

Your evidence is very good that zinc is bound to sulfhydryl groups from the *p*-chloromercuribenzoate (PCMB) and silver experiments; and, yet, you apparently did not displace the zinc from the intact enzyme by PCMB but took it off by other means. Is there any other metal-sulfhydryl bond in a protein where the metal is not displaced by PCMB or, as you pointed out, by other similar reagents?

DR. VALLEE: We did replace zinc with copper (see Table 2). I am not aware that experiments in which metals were displaced from sulfhydryl groups with PCMB have been performed and validated analytically, so that I cannot make comparisons. However, if PCMB or silver would displace zinc from carboxypeptidase the difference in the titration of an —SH group in the metal-free and the zinc containing enzyme would not be apparent: one —SH group would then be titrated in both cases.

DR. SCHUBERT: I would just like to comment on the problem of probing for sensitive spots in the protein. Actually, one can use metals themselves. For example, zinc may be used to prove whether you have a sulfur group as you have shown. An even more specific example can be taken from our results with serum alkaline phosphatase, which is strongly inhibited by beryllium (Schubert, J. and A. Lindenbaum. *J. Biol. Chem.* 208: 359, 1954 and Lindenbaum, A., M. R. White and J. Schubert. *Arch. Biochem. & Biophys.* 52: 110, 1954). We have found that beryllium reacts under physiological conditions only when a phenolic group is present suggesting that tyrosine must be present in this enzyme. Hence, by using selective combinations of metals one can probe the structure of the functional groups of a protein or enzyme. Instead of using organic agents, one uses the metals themselves.

DR. VALLEE: I am very glad that you mention this circumstance. It is precisely along the lines which I wanted to emphasize. "Metals as specific protein reagents represent a field that has barely been suggested but not as yet explored," to quote Fraenkel-Conrat (Fraenkel-Conrat, H. In: *The Enzymes*, Vol. 1, 2nd ed. edited by P. D. Boyer, H. Lardy, and K. Myrbäck. New York: Acad. Press, 1960, p. 589). Carboxypeptidase which I discussed and the inhibition of alkaline phosphatase with beryllium might be considered different approaches to the same problem.

DR. ALBERT: Is it still believed that alcohol dehydrogenase of liver is free from mercapto groups, whereas that of yeast is rich in them?

DR. VALLEE: Yeast alcohol dehydrogenase has been studied more extensively in this regard and I shall, therefore, discuss this enzyme first. Dependent upon the methods and conditions employed, from 24 to 36 —SH groups can be titrated in YADH. It is clear that —SH groups are related to the activity of the enzyme though the *manner* in which they participate is unknown. Nor has it been possible to delineate the simultaneous participation and essentiality of the zinc atom in enzymatic activity. The studies reported here and previously (Snodgrass, P. J., B. L. Vallee, and F. L. Hoch. *J. Biol. Chem.* 235: 504, 1960; Kägi, J. H. R. and B. L. Vallee. *J. Biol. Chem.* 235: 3188, 1960) illuminate the problem and suggest that zinc is bound to sulfur.

There is much less information on the number of —SH groups in alcohol dehydrogenase of horse liver. Using an argentometric method we have been unable to obtain reproducible data.

DR. HASTINGS: I want to take a little umbrage with Dr. Vallee's statement that zinc has pursued him. It has pursued him in the same way that an enraged lion would pursue a

hunter on safari. About 16 or 17 years ago I encountered Bert at the Harvard Medical School and he said, "How can I get an emission spark spectrum of white cells? I am looking for the metals they contain." This is the point where he entered the jungle—he entered the lion's den. Seriously, I think that he and his colleagues have carried out beautiful work. My question is: Do you think the fact that there is a DPN requirement for the alcohol dehydrogenase has anything to do with the fact that you can't put the zinc back and restore activity?

DR. VALLEE: It is a reasonable presumption that zinc may be bound to two sulfur groups (Kägi, J. H. R., and B. L. Vallee. *J. Biol. Chem.* 235: 3188, 1960). We are postulating that the removal of zinc results in the oxidation of the two sulfur groups to which, we presume, it to be bound, and the reintroduction and reactivation are prevented thereby. The oxidation of the —SH group in carboxypeptidase prepared from pancreatic juice gives support to this idea, since it is oxidized almost instantaneously on zinc removal. In contrast, the —SH group in the enzyme prepared from acetone powder oxidizes very much more slowly, permitting conjoint restoration of zinc and activity.

DR. COTZIAS: Dr. Snell, would you say that the phylogenetic tendency is for the body to get rid of metals or to employ them better as time goes on?

DR. SNELL: Do you want my prejudices in connection with this? I would hope that we would find more efficient ways of carrying out metabolic processes as evolution continues. Whether this involves becoming free of the requirement for metals or relying on them to an even greater extent, I certainly couldn't say.

DR. VALLEE: I should like to congratulate Dr. Snell on his very interesting discussion of another aspect of metal and enzyme interactions. In amplifying his remarks I should point out that the activation of enzymes by different metal ions takes varied forms which have been discussed also by Dr. Williams (Williams, R. J. P. *Biol. Revs. Cambridge Phil. Soc.* 28: 381, 1953; Williams R. J. P. and B. L. Vallee. *Discussions Faraday Soc.* No. 20, 262, 1955). We have studied a type of interaction yet different from those here described by Dr. Snell and from others discussed in the literature. The succinic dehydrogenase cytochrome c reductase system of mitochondria is activated by ATP, magnesium and manganese and is inhibited by calcium (Hoch, F. L., H. R. Tyler and B. L. Vallee. *Fed. Proc.* 17: 243, 1958. See also Vallee, B. L. In: *The Enzymes*, Vol. 3, pt. B. edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1960, p. 225). The enzymatic response to ATP and to the pairwise addition of magnesium and manganese on one hand and to magnesium and calcium on the other is not that which would be expected from the sum of the effects of the metals; rather a complex interaction between them is demonstrated. In this instance, as in those just described by Dr. Snell, the response of activity to the addition of activating metals, one at a time, may give an adequate view of the chemical reaction mechanisms involved. However, in our experiments it is difficult to decide, of course, which metal may be that one which dominates the *biological* effect. The elegant experiments of Dr. Snell are important steps in approaching the controlling effects of metal ions in metabolic processes.

The importance of H^+ ion concentration as a variable in the control of enzymatic mechanisms by metals is well illustrated by the effect of pH on metal and activity restoration of carboxypeptidase A (Coleman, J. E. and B. L. Vallee. *J. Biol. Chem.* 235: 390, 1960).

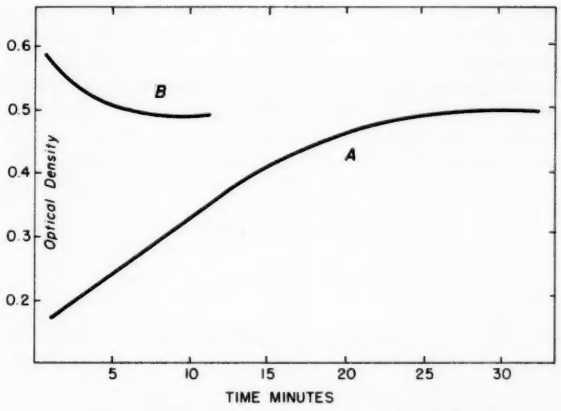


FIG. 1. Formation of nickel-salicylaldehyde-glycine.

DR. CHRISTENSEN: It might be of interest to crystallographers that these chelates mentioned by Dr. Snell crystallize very readily. I know that there is current interest in crystallographic studies of them. Many such chelates of Mn^{++}, Zn^{++}, Fe^{++}, Fe^{+3}, etc. can be obtained readily.

DR. EICHHORN: Dr. Snell mentioned the fact that the metal in the pyridoxal amino acid complex can function in a number of possible ways. One of these is to bring the amino acid and pyridoxal together. Another is for the metal to serve as a catalytic agent. We have performed an experiment which may be construed as evidence that the latter rather than the former is the function of the metal. We have taken salicylaldehyde and reacted it with glycine in the presence of nickel and followed the conversion of the nickel-glycine spectrum to the nickel-salicylaldehyde-glycine spectrum. Nickel-salicylaldehyde-glycine absorbs much more strongly at 900 mμ than nickel glycine so that the reaction can be followed at this wave length. The curve for the reaction in which salicylaldehyde is added to nickel-glycine is shown in the figure as curve A. If one starts out with salicylaldehyde and glycine in solution and then adds nickel and follows this reaction, one gets the curve shown as B. The spectrum of the final product, whether one starts with nickel-glycine and adds salicylaldehyde, or whether one starts with salicylaldehyde-glycine and adds nickel, is exactly the same. This spectrum is presumably that of nickel-salicylaldehyde-glycine in equilibrium with a small amount of nickel-glycine. It appears from the figure that the Schiff base present before the addition of nickel forms a complex with nickel, and that, subsequently, a portion of this complex decomposes into nickel-glycine. It, therefore, appears that the metal is not very effective in bringing the two components of the Schiff base together but probably functions in the second of Dr. Snell's alternative ways.

DR. WILLIAMS: I am not sure the point I would like to make is clear to the biologists and biochemists who are discussing some of the problems in association with metal ion activation of reactions. This point is that a metal ion carries a positive charge and this can be one of the ways in which it activates. In coordination chemistry people often write pothooks and arrows to show that the electron can drift toward a positive charge and this is the essential activation step. One should not

be surprised if, under certain circumstances, a reaction which goes with one positive charge can be activated by another positive charge. For example, a series of metal ions will activate a given process with a range of activity. One might say that it is surprising that the proteins which can carry out a reaction in some living organism require a metal but not in others. It is not surprising because there is another metal ion which is H^+. The proton can often carry out much the same function, so far as catalysis is concerned, as any other cation.

DR. SNELL: Dr. Williams' point is certainly valid. We pointed out this possible function for the metal ion in our original paper on the mechanisms of these nonenzymatic reactions, published in 1954, and, also, that this same function might be filled by proteins in the enzymatic reactions *in vivo*.

The living cell as a metal-binding agent

HALVOR N. CHRISTENSEN

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

At this stage in our conference I am to introduce the living cell as a metal-binding agent. This may be an embarrassing introduction.

First of all, the cell presents *surface* binding sites, which often behave much like the various complexing agents that have been discussed so far. If the cell also presents a reasonably effective barrier retarding deeper penetration, one may be able to study such surface binding rather clearly, as Aser Rothstein has done for yeast cells. In many cases, these barriers are, however, eventually penetrated. No doubt transient metal-binding sites are encountered on the way, which will tend to give up the metal ion again to deeper agents that bind more stably. If any class of such transient binding sites *must* be occupied before the metal can penetrate deeper, or if combination with such sites facilitates further penetration under appropriate conditions, we can designate them as *transport sites*.

It is this interesting barrier region, and the events that occur therein, that I will most enjoy discussing in making this introduction. These are the portals of the cell, through which many solutes pass with great speed and sometimes appear to be impelled, while other smaller ones, especially cations, are largely restrained from passage. The consequence of this situation is that the passage of metal ions may well occur more as a result of sequential complexing or the movements of mobile chelating agents and mobile metal complexes than by the free movement of the metal ions themselves.

Experiments with yeast. But let us first go back to the cell surface and examine the interesting experiments of Rothstein and his associates. The ion-binding properties of yeast cells were first studied with uranyl ion (5). These cells proved to be able to remove small quantities of uranyl ion almost entirely from the environmental solution. But as the level of uranyl ion was increased the cells became saturated at a rather low level, about 10 million uranyl ions per cell (or 1 millimole per liter of cells). With higher concentrations the uptake increased only slightly beyond this point. This binding was fully reversible. The position of the fixed uranyl ion was taken to be superficial because inorganic phosphate added to the medium removed some of the cation, presumably by competitive complex formation; yet the cell cytoplasm contains inorganic phosphate at a level 50 times as high as that outside. The implication is that the metal ion is bound outside the barrier separating high and low phosphate levels, namely the plasma membrane. Its position in relation to the more superficial *cell wall* is not established by this experiment. The behavior of the binding groups on the cell surface suggested that the binding groups were phosphate residues.

Other cations such as Mn^{++} were able at relatively high levels to compete with uranyl ion for these surface sites. The behavior was highly analogous to that of a cation-exchange resin. A mass-law plot of the Mn^{++} binding revealed a second class of binding sites, which were taken to be the carboxyl groups of proteins. The anionic sites on the cell appeared to be so far apart that they behaved independently; a 1 to 1 competition was obtained between univalent and divalent cations.

In the resting yeast cell (no substrate) external divalent cations did not exchange with those in the cytoplasm. In the presence of glucose and phosphate, however, additional quantities of Mg^{++} or Mn^{++} were taken up, and if K^+ was also added, the uptake was further increased. Mn^{++} levels used were of the order of 1 millimolar. Mn^{++} uptake was not observed in the absence of phosphate, although, conversely, phosphate uptake occurred unhindered in the absence of Mn^{++} (6). Arsenate interfered with Mn^{++} entrance of the cells. A considerable specificity to Mg^{++} and Mn^{++} was shown; Ca^{++} uptake was only one-tenth as great, UO_2^{++} uptake very small. Although the uptake of phosphate and Mn^{++} appeared under some conditions to be proportional, the uptake of phosphate could precede that of Mn^{++} by some hours. This increased absorptive capacity for Mn, engendered by preceding phosphate uptake, gradually decayed, the loss being accelerated when sugars were fermented. The investigators suggest that a carrier for Mn uptake is synthesized within the cell membrane as a direct product of the phosphate-transfer system (2). The stimulation by K^+ uptake is believed to arise indirectly by the facilitation of phosphate uptake caused by this ion. It may be noted that there are no indications that Mn is transferred against a genuine concentration gradient or that the carrier operates reversibly. Under the conditions, Mn^{++} or Mg^{++} accumulate in a compartment where they are no longer readily exchangeable with ions in the external medium; they may well be in complexed forms here also. Therefore, this carrier does not necessarily have the complex attributes required, say, for the cellular K^+ carrier; perhaps the Mn^{++} "carrier" may only be a *bridge*.

The cations fixed on yeast cells bear another interesting relationship to the cell barrier. If uranyl ions are fixed, the specific transport process for *glucose* is inhibited, but when Mn^{++} or Mg^{++} are fixed, glucose uptake is enhanced. These results seem to indicate that the metal-binding site is part of the glucose transport machinery. On the other hand, when the superficial sites are filled by the presence of uranyl ions in excess, the uptake of Mn^{++} continues undisturbed. Hence these sites do not need to be occupied by Mn^{++} as an essential stage in its transfer through the barrier; that is, they do not operate as Mn^{++} transport sites, nor do they operate as K^{+} transport sites.

These latter results recall the long-known ability of Cu^{++} at 10^{-7} M, or of Hg^{++}, to block reversibly the chemically mediated transfer of glycerol into human red blood cells (1). Cu^{++} needs to occupy only a very few widely-separated sites on the cell to block the mediation. Stein showed recently that the presence of free histidine, or 0.17 N Cl^{-}, suffices to relieve the Hg inhibition, indicating that the site involved binds the metal rather weakly (9). Treating the red cell stroma with fluorodinitrobenzene or with phenylisothiocyanate permitted Stein to isolate in comparatively small quantities an N-terminal histidine. Furthermore, the presence of 1,3-propanediol, a competitive inhibitor of glycerol transport, blocks the reaction of this particular histidine residue with phenylisothiocyanate, a behavior not shown by the N-terminal histidine in gliadin. Accordingly, Stein suggests that the metallic ion binds to an N-terminal histidine, which is part of the glycerol transport site. Mercury also blocks the specific transport of glucose into the human red blood cell.

These results bring out an interesting association of the exposed chelating sites on the cell surface with sites functioning in the transport of other solutes.

Experiments with slices. These may be illustrated from the work of Saltman *et al.* with metal uptake by liver slices (7, 8). At approximately a 0.5 mM level, the net uptake of iron and the uptake of labeled ferric iron proceeded at about the same rate. On the other hand, the accumulated iron was lost much more quickly into iron-containing than into iron-free solutions. This paradox probably arises because the Fe^{+3} concentration to which the slices were exposed is so abnormally high that in comparison there is, to begin with, little iron in the tissue available for exchange. Once iron had been taken up to an increased level its exchangeability became evident.

Iron uptake was not dependent on metabolic energy; the iron was taken up by sites subject to saturation, diffusion to those sites being rate-limiting. The uptake rose with temperature because more sites became available; fixation reached a maximum between 50 and 60°. Freezing and thawing the tissue produced no acceleration of uptake nor did treatment with surface-active agents. If these treatments really broke the cells, the implication is that no otherwise inaccessible binding sites were exposed by cell rupture. Related results have been reported for other metals.

Tissue slices appear to present the most serious problem of distinguishing extracellular from cellular fixation and in identifying the barriers that retard metal entrance.

These experiments introduce also the complex problem of exchange *versus* net transfer. We know that chelates of similar stability vary a great deal in the rate at which the metallic component exchanges with free metal ion and that a chelate that yields little metal ion as a net by dissociation may exchange its metal ion with great speed with a pool of the free metal ion. Such exchange would not be taken to measure the stability of the chelate.

We are also becoming familiar with the perhaps related biological phenomenon that the flux of a labeled solute from a cell may be accelerated by the presence of a high concentration of that solute or an analog in the external medium. Or, conversely, as Heinz and Walsh showed, the influx of labeled glycine *into* the Ehrlich cell is increased in proportion to the level of unlabeled neutral α-amino acid already accumulated. An additional example of this phenomenon has just been reported in a preliminary presentation by Lajtha and Toth (3). The amino acid levels of the cerebrospinal fluid are not readily increased by elevating the plasma amino acids. A rapid exchange occurs, however, between these compartments, and this exchange is promptly accelerated by raising the plasma amino acids.

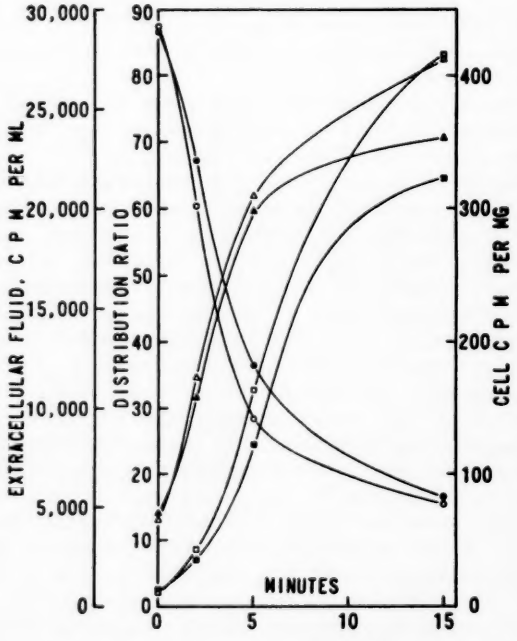


FIG. 1. Effect of previously accumulated diaminobutyric acid on uptake of Mn^{54} by Ehrlich ascites tumor cells. Open figures show the uptake by the unloaded cells, the closed figures the uptake by cells previously brought to a steady state with 10 mM diaminobutyrate. Squares represent distribution ratios, triangles the radioactivity gained by the cells, and circles the residual radioactivity of the external medium. (Reproduced with permission from Pal, P. R. and H. N. Christensen. *J. Biol. Chem.* 234: 615, 1959.)

The occurrence and specificity of such exchanges may be very informative as to the nature of the connecting linkage between the cell, tissue, or organism and its environment. The possibility that such concentration-responsive exchanges may be unaccompanied by net migrations must strongly influence, however, the physiological interpretation of changes in the distribution of an isotopic tracer.

Experiments with free-cell tumors. Experiments were performed with the Ehrlich ascites tumor cell by Dr. Prabhat Pal in our laboratory, in an effort to determine whether a metal ion might be involved as a carrier in amino acid transport (4). This possibility had been suggested by the sensitivity of transport to the spacing between the amino and carboxyl group, and for diamino acids, between the two amino groups. Also, the sensitivity of amino acid transport to ethylenediaminetetraacetate and 8-hydroxyquinoline, and in bacteria, to Mg^{++} and Mn^{++} depletion, had supported the same possibility. In this study we followed the uptake of the radioactive isotopes of the metals. Carrier-free $(Mn^{54})^{++}$, for example, was supplied at such a low level that most of the Mn in the suspending medium must have been that released from the cells. The uptake of this isotope was characterized by a rapid phase of about 15 minutes and then a slow phase continuing indefinitely. The behavior was rather similar at 0.5 mM Mn. A part of the fixed Mn was eluted immediately when a cell suspension was diluted with ice-cold Krebs-Ringer bicarbonate, other portions being released only very slowly.

The objective of these experiments was not to study metal ion uptake *per se* but to determine whether simultaneous amino acid uptake led to accelerated metal influx. If a metal ion were serving as the carrier such a flux association might be anticipated. Actually, it appears not to be necessary to begin with the amino acid on the outside of the cell in such an experiment, because accumulated amino acids are subject to a steady leak from the cell, and cells heavily loaded with an amino acid will be reaccumulating the escaping amino acid at a steady rate. Indeed, this situation may provide an optimal opportunity for testing the hypothesis; if we begin instead with all of the test amino acid on the outside, any acceleration of metal influx could be masked by a chelation of part of the metal by the amino acid in a nonpermeating form in the external phase. We do not need to be concerned that the mere presence of additional chelating amino acid within the cell will accelerate the uptake of the metal, because the metal entrance is not limited by the number of binding sites available inside the cell. When the Ehrlich cell is broken, the rate of binding of metals to the sedimentable residue is enormously increased.

TABLE 1. *Effect of the Presence of 10 mM Diaminobutyric Acid on Uptake of Radioactive Zn^{++}, Cu^{++}, and Fe^{+3} by Ascites Cells During 1 Hour**

Metal	Concentration, mM/L	Cell Uptake, cpm/mg		Distribution Ratio	
		No addition	Plus amino acid	No addition	Plus amino acid
Cu	0.0017	63.8	27.6	3.2	1.2
Cu	0.13	83.4	11.7	6.9	0.6
Fe	Trace	41.7	36.9	4.1	3.8
Zn	0.0001	273	263	22.7	18.6
Zn	0.05	126	71.7	9.8	3.8
Zn	0.5	225	85.2	29.8	3.8

* Pal, P. R. and H. N. Christensen. *J. Biol. Chem.* 234: 615, 1960.

TABLE 2. *Effect of the Presence of L-Cysteine and D- and L-Penicillamine (all 10 mM) on Uptake of Mn^{54} (Tracer Level) by Ascites Cells During 1 Hour*

Amino Acids	Cell Mn^{54} cpm/mg		Distribution Ratio	
	No addition	Plus amino acid	No addition	Plus amino acid
L-Cysteine	306	282	59.5	32.8
L-Penicillamine	277	96.2	49.0	4.9
D-Penicillamine	287	29.2	52.5	1.3

Figure 1 shows how the uptake of Mn^{54} at tracer levels is accelerated when the tumor cells have been preloaded with α,γ-diaminobutyric acid. Perhaps the lower curves, marked by squares are the most informative; they show the rising partition coefficient for Mn^{54}. The other curves show rising cell radioactivity and the declining extracellular activity. The open figures mark the result in the presence of diaminobutyrate. Lysine and arginine also show this effect, but it is not noticeable with several neutral amino acids. The stimulation persists as the Mn level is increased to 0.5 mM. The extra Mn fixed was mainly not of the easily eluted character.

What may be interesting to the present conference is that $(Fe^{59})^{+3}$, $(Cu^{64})^{++}$, and $(Zn^{65})^{++}$ uptakes by these cells are only decreased, especially for the latter two, by the presence of diaminobutyric acid (Table 1). Furthermore the strong chelators cysteine and penicillamine (Table 2) only interfere with the uptake of Mn. D-Penicillamine is especially inhibitory to Mn uptake perhaps because more of it remains outside the cell. We may conclude that the stimulation is somewhat specific as to the nature of both the chelator and the metal ion. Perhaps Dr. Cotzias' remarks will help us to understand this specificity for Mn.

Perhaps we may conclude that diaminobutyrate can serve as a carrier for Mn with much more certainty than any decision about the role of Mn in diaminobutyrate transport can receive. Perhaps the specificity of amino acid transport to α-amino acids arises from spacings in the membrane matrix which are bridged by the NH_2—CHR—COO^- structure, with the two functional groups bonding to polar sites, according to the polar-pore theory of Stein and Danielli (10). Perhaps the failure of metal ions to accompany the amino acids reveals that the coordinating groups are taken up so as not to be available for complexing to the metal. The association of the Mn influx with basic amino acid uptake could then be a con-

sequence of the availability of at least the second basic group for coordination during transport. A metal, under this hypothesis, would need to release the α-amino group (which Cu and Zn might fail to do) to permit the complex to migrate. Coordination to membrane sites might also occur. Thus, metal ion fluxes might serve to reveal what functional groups on transported solutes need not participate in the transport reaction. Dr. Albert has suggested in a thoughtful letter that the acceleration of the Mn flux might mean that lysine or diaminobutyrate are phosphorylated during transport. Phosphorylation of the amino acids appears, however, to require a prohibitive amount of energy in view of the rapid amino acid exchange that occurs.

REFERENCES

1. JACOBS, M. H. AND S. A. CORSON. *Biol. Bull.* 67: 325, 1934.
2. JENNINGS, D. H., D. C. HOOPER AND A. ROTHSTEIN. *J. Gen. Physiol.* 41: 1019, 1958.
3. LAJTHA, A. AND J. TOTH. *Fed. Proc.* 19: 130, 1960.
4. PAL, P. R. AND H. N. CHRISTENSEN. *J. Biol. Chem.* 234: 613, 1959.
5. ROTHSTEIN, A. In: *Electrolytes in Biological Systems*, edited by A. M. Shanes. Washington, D. C.: Am. Physiological Soc., 1955, p. 65.
6. ROTHSTEIN, A., A. HAYES, D. JENNINGS AND D. HOOPER. *J. Gen. Physiol.* 41: 585, 1957.
7. SALTMAN, P., R. D. FISKIN AND S. B. BELLINGER. *J. Biol. Chem.* 220: 741, 1956.
8. SALTMAN, P., H. L. FRISCH, R. D. FISKIN AND T. ALEX. *J. Biol. Chem.* 221: 777, 1956.
9. STEIN, W. D. *Nature, London* 181: 1662, 1958.
10. STEIN, W. D. AND J. F. DANIELLI. *Discussions Faraday Soc.* 21: 238, 1956.

DISCUSSION

Time dependent activation of phosphoglucomutase by magnesium and imidazole

JOHN P. ROBINSON AND VICTOR A. NAJJAR

Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee

THE ROLE OF METALS in enzyme reactions may involve the interaction of the metal with the enzyme to form the metalloenzyme. In this instance, the metal acquires permanent, semipermanent, or temporary residence within or on the molecule, depending on the extent of dissociation. It may also interact with the substrate to form the metallosubstrate, the true substrate of the reaction (9). In both instances, the metal should be necessary for the overall reversible catalysis. Failing this, the metal cannot be considered a functional part of the system. A pertinent example is the effect of Mg on glucose 1-phosphate transphosphorylase (glucose 1-phosphate $\rightleftharpoons$ glucose + glucose 1,6-diphosphate). Mg strongly stimulates the forward reaction. However, it inhibits the reverse reaction equally strongly. Such an effect is due to the displacement of equilibrium through the formation of Mg-glucose diphosphate complex (14).

We have lately investigated the role of Mg in the activation of phosphoglucomutase (12). This system is well suited for such a study, since Mg activation is time dependent and the mechanism of action of the enzyme well elucidated (10). Catalysis proceeds in two steps

$$\begin{array}{lcccc} & \text{glucose 1-phosphate} & + & \text{phosphoenzyme} & \\ 1. & \downarrow\uparrow & & & K = 4.58 \\ & \text{glucose 1,6-diphosphate} & + & \text{dephosphoenzyme} & \\ 2. & \downarrow\uparrow & & & K = 3.76 \\ & \text{glucose 6-phosphate} & + & \text{phosphoenzyme} & \end{array} \qquad (13)$$

Thus two forms of enzyme are necessary for catalysis, the phospho- and dephospho-forms. It was particularly important to determine the role of Mg with respect to these two forms of enzyme. Crystalline enzyme preparations were used throughout this study (8).

Phosphoenzyme. Preincubation of the enzyme with Mg and imidazole (or histidine) at pH 7.5 results in a 5–6-fold increase in the rate of catalysis. By contrast, no measurable activation occurs during enzymatic activity, notwithstanding the necessary presence of both Mg and imidazole. In all cases activity measurements were made under standard conditions of glucose 1-phosphate 4×10^{-3} M, Mg 1×10^{-3} M, imidazole 4×10^{-2} M, pH 7.5 and 30°. It was found more convenient to carry out activation by preincubation at 0° since it requires 5–10 minutes to reach maximum. Mg alone or imidazole alone at

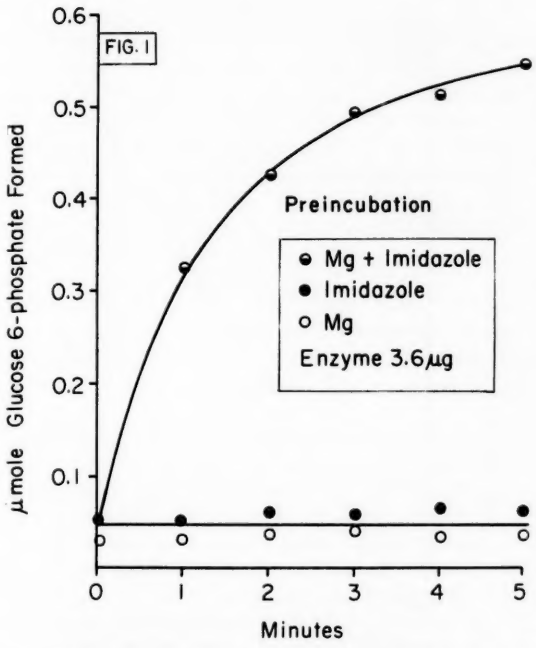


FIG. 1. Effect of preincubation of the phosphoenzyme at 0° in Mg alone, imidazole alone and Mg with imidazole. At the preincubation times indicated, the appropriate amount of enzyme shown was assayed under standard conditions as follows: 0.5 ml of reaction mixture containing glucose 1-phosphate 4×10^{-3} M, Mg 1×10^{-3} M, imidazole 4×10^{-2} M, at 30°. The amount of glucose 6-phosphate formed in 1 minute is recorded on the ordinate and represents the degree of activation attained with preincubation.

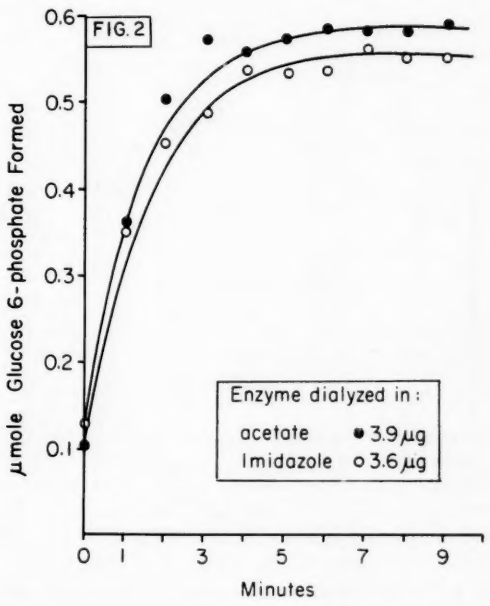


FIG. 2. Similar activation kinetics exhibited by preparations of phosphoenzyme dialyzed for 20 hours in acetate buffer 0.15 M pH 5.0 or in imidazole buffer 0.04 M pH 7.5. Activation rates for undialyzed enzyme fell within the same range. Details are the same as in Fig. 1.

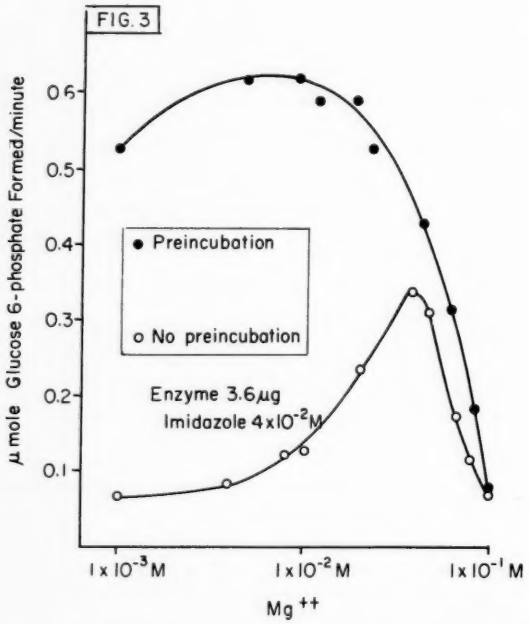
0.04 M or less produced no measurable activation (Fig. 1). However, imidazole alone at 0.4 M produced significant activation possibly due to minimal Mg contamination. Mn and Co even with imidazole produced little or no activation. It appears that the activator is a Mg-imidazole complex since both are necessary and an increase in one or the other, within limits, causes increased activation. Thus with Mg 1×10^{-3} M the activation in imidazole 4×10^{-2} M is about five times faster than that obtained in imidazole 4×10^{-3} M. Conversely, at imidazole concentration of 4×10^{-2} M the activation with Mg 1×10^{-2} M is about twice that with Mg 1×10^{-4} M and seven times that obtained in Mg 1×10^{-5} M. A similar activating effect on aconitase by Fe^{++} and cysteine has been reported (5).

It is unlikely that activation under these circumstances is due to removal of inhibitory metals since prolonged dialysis in imidazole 0.04 M pH 7.5 or acetate 0.15 M pH 5.0 at 4° does not alter the magnitude or characteristics of activation (Fig. 2).

The manner by which Mg and imidazole bring about this slow activation is not clear. It is tempting to assume that imidazole functions to allow effective diffusion of Mg ions deep into the active site of the molecule. On this basis, it should be possible to measure a slow rate of deactivation upon subsequent exposure of the activated enzyme to imidazole alone. It should also be possible to effect some measure of activation by very prolonged incubation with Mg alone. As it turns out, neither can be accomplished. When maximally activated enzyme, in Mg 1×10^{-3} M and imidazole 4×10^{-2} M, was diluted with imidazole such that final Mg concentration fell below 1×10^{-5} M, there was a drop to preactivation level of catalysis within 30 seconds after dilution. Controls were diluted in Mg and imidazole. Furthermore, prolonged incubation or dialysis of the enzyme against Mg 1×10^{-3} M at 4° for two days does not result in any activation. The addition of 0.04 M imidazole to the same preparation results in maximal activation within 10 minutes.

Other features of the activated enzyme are of interest. Like the nonactivated enzyme, it requires optimal concentrations of Mg and imidazole. Neither alone will suffice. Figure 3 shows the effect of varying concentrations of Mg on the activated and nonactivated enzyme under otherwise standard conditions. It can be seen that the activated enzyme has a broad optimum with a maximum at 4×10^{-3} M. This contrasts with the sharp maximum at 6×10^{-2} M Mg for the nonactivated enzyme. In fact, at this latter concentration the activated enzyme shows considerable inhibition. Further, at the level at which the activated enzyme shows maximum activity, the nonactivated enzyme shows minimal rate of catalysis.

The optimal concentration of Mg required in the reaction mixture varies with the concentration of imidazole



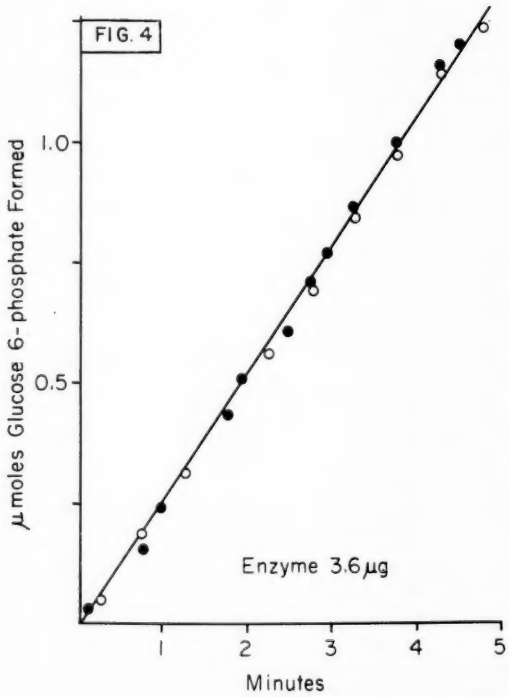


FIG. 3. Effect of various concentrations of Mg on the catalytic activity of maximally activated and nonactivated phosphoenzyme at otherwise standard conditions detailed in Fig. 1.

FIG. 4. Absence of activation during enzyme catalysis. Two experiments are shown with nonactivated phosphoenzyme and performed at standard conditions as detailed in Fig. 1. The amount of glucose 6-phosphate formed at the times indicated is recorded on the ordinate. Had there been any measurable activation during the reaction, an autocatalytic type of curve would have been obtained.

and vice versa. This indicates that here too a Mg-imidazole complex is responsible for this phenomenon. It appears then that not only does the complex activate the enzyme, but also that its presence during catalysis is mandatory for the maintenance of the activated state. It became of interest, therefore, to investigate the quantitative composition of this complex.

On the basis that the rate of catalysis is proportional to the concentration of Mg_1 (imidazole)$_1$ complex, which in turn is directly proportional to the product of the concentrations of Mg and imidazole, the following relationships should hold [Mg] $\times$ [imidazole] $\times$ K = [Glucose 6-phosphate] obtained per minute (as an expression of the rate of catalysis). The nonactivated form proved more suitable for this study because of its sharp activity response to convenient concentrations of Mg (Fig. 3). As an example, we observed that with 2×10^{-2} imidazole, Mg concentrations of 8×10^{-3} M, 1×10^{-2} M and 4×10^{-2} M gave K values of 2.5×10^{-1}, 3×10^{-1}, and 2.6×10^{-1}, respectively, and at 4×10^{-2} M imidazole K values were 1.8×10^{-1}, 2.3×10^{-1}, and 2.4×10^{-1}, respectively. A constant K was not obtained, however, when Mg_1 (imidazole)$_2$ was considered. The values for K varied from 2.2×10^{-4} to 6.1×10^{-2}.

The same type of complex is presumed to be responsible for activation during preincubation with Mg and imidazole. This has not yet been investigated since the nature of the activation response does not lend itself readily for this type of study.

It has been noted earlier that preparations of enzyme, not subjected to activation, do not show any measurable activation during catalytic activity. Figure 4 shows this type of behavior. Nonactivated enzyme was added to a standard reaction mixture and at the times indicated samples were obtained for assay of glucose 6-phosphate formed (12). The constant rate shown for a period of 10 minutes indicates the lack of any activation. This contrasts with the rapid activation during the first 30 seconds of preincubation. Since the preincubation mixture differs from the reaction mixture only in the absence of substrate, it is clear that the substrate in some manner prevents the activation of the enzyme. From the mechanism of the reaction and the equilibrium constants of the two steps, it would be expected that under standard conditions of the reaction most of the enzyme would be in the dephospho-form. A study of the activation of this form, and the possible effect of substrate on this process, was therefore indicated.

Dephosphoenzyme. Preparations of this form of enzyme were made as before. Phosphoenzyme 0.5–1.0 μmole was reacted 3–4 times with 10 μmoles of glucose 6-phosphate under standard conditions, followed by 8 hour dialysis

against repeated changes of 0.04 M imidazole at 6°. After the final treatment dialysis was continued for 20 hours (13). Such preparations were usually found to be maximally active or would reach maximum activation within 30 seconds. Some preparations were found to be activable much like the phosphoenzyme. It is possible that such preparations still contained minimal amounts of glucose 6-phosphate. When dephosphoenzyme is preincubated in Mg and imidazole with 4×10^{-6} M glucose 1- or 6-phosphate the catalytic rate was 20–40 per cent of the controls lacking the substrate. It thus appears that the suppression of activation during enzymatic reaction can be explained by the effect of the substrate on this enzyme form. In this respect, it is similar to the inhibitory effect of substrate on the Mg activation of phosphatase (2–4).

To explore other possible differences between the two forms, we have lately made preliminary observations on the optical characteristics of the two forms of the enzyme. Enzyme preparations crystallized two times were used. There was no difference in the extinction at 280 mμ between the two forms. However, we observed a consistent difference in optical rotation studies. Phosphoenzyme and dephosphoenzyme were obtained as described above (13). All preparations were thoroughly dialyzed at 6° in imidazole buffer 4×10^{-2} M pH 7.5 in preparation for optical measurements. These were made at enzyme concentrations of about 30 mg per ml at 22°. The Keston attachment to the Beckman spectrophotometer, calibrated with sucrose, was used throughout. The absolute values will eventually be measured with more conventional instruments. However, we have recorded tentative values for phosphoenzyme $[\alpha]_D = -51°$, and dephosphoenzyme $[\alpha]_D = -47.8°$. Furthermore, with each of the six different preparations studied so far, there was an immediate and stable shift to less negative values when most of the phosphoenzyme was converted to the dephospho-form by the addition of glucose 6-phosphate. In these cases, optical rotation at 589 mμ was recorded for the phosphoenzyme then followed by the addition of 4 μ moles of glucose 6-phosphate per milliliter of enzyme. This immediately converts about 75 per cent of the enzyme to the dephospho form. Optical rotation was then measured a few minutes following this treatment. After correcting for the contributions of the glucose phosphates present at equilibrium, there was obtained a shift of about 2.0° towards the positive. Further addition of glucose 6-phosphate produced no measurable effect. No change in optical rotation was observed when glucose 6-phosphate was added to dephosphoenzyme under similar conditions. It is, of course, necessary to ascertain whether a similar magnitude of change towards the negative is obtainable when dephosphoenzyme is converted to the phosphoform. Glucose 1,6-diphosphate is currently being synthesized for that purpose (11).

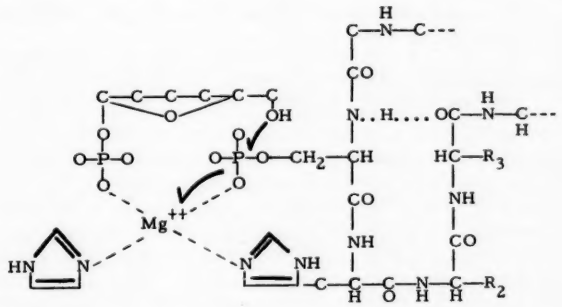


FIG. 5. This illustrates the postulated enzyme-substrate transition complex. Histidine may be in a phosphoserylhistidine sequence. It may well be on a neighboring chain instead, but with the proper orientation and geometry.

The observed difference in optical rotation between the two forms of the enzyme and the role of Mg in activation and catalysis cannot be readily interpreted with any degree of comfort at this time. However, one is tempted to put together what is already known about the enzyme into an entertaining though not durable scheme. The enzyme has one phosphate group per molecule which is completely transferable during activity (13) and like many phosphoproteins, the phosphate has been shown to be a serine phosphate (1). The importance of histidine for the activity of the enzyme (6) received added emphasis by the suggestion that a phosphoserylhistidine dipeptide is at the active center (7). Based on this, one can picture the role of Mg as forming a complex involving the imidazole of histidine and the phosphate of serine. The strong positive charge of Mg^{++} would serve to lower the electron density at the serine phosphate bond, thereby labilizing that bond and facilitating the transfer of phosphate to glucose phosphate. The loss of phosphate would then allow the formation of a helical structure with hydrogen bonding involving the N—H of the serine. This otherwise is denied by the covalent phosphate, which tightens the serine N—H bond, thus preventing hydrogen bonding with the —C=O of the appropriate residue. This is perhaps an over interpretation of the small optical rotation change observed which might well result from factors unrelated to helical conformation. Further work on the optical parameters, sedimentation characteristics and intrinsic viscosity is indicated for more meaningful interpretation.

REFERENCES

1. ANDERSON, L. A. AND G. R. JOLLES. *Arch. Biochem. Biophys.* 70; 121, 1957.
2. CLOETENS, R. *Biochem. Ztschr.* 307: 352, 1941.
3. CLOETENS, R. *Biochem. Ztschr.* 308: 37, 1941.
4. CLOETENS, R. *Biochem. Ztschr.* 310: 42, 1941.
5. DICKMAN, S. R. AND A. A. CLOUTIER. *J. Biol. Chem.* 188: 379, 1951.
6. KOSHLAND, D. E., JR., J. R. WILLIAM AND M. J. ERWIN. *Fed. Proc.* 17: 1145, 1958.
7. MILSTEIN, C. AND F. SANGER. *Biochim. et biophys. acta* 42: 173, 1960.
8. NAJJAR, V. A. *J. Biol. Chem.* 175: 281, 1948.
9. NAJJAR, V. A. In: *Phosphorus Metabolism*, vol. I, edited by

W. D. McElroy and H. B. Glass. Baltimore: Johns Hopkins Press, 1951, p. 500.
10. Najjar, V. A. and M. E. Pullman. *Science* 119: 630, 1954.
11. Posternak, T. *Methods in Enzymology*, vol. III, edited by S. P. Colowick and N. O. Kaplan. New York: Acad. Press, 1957, p. 147.
12. Robinson, J. P. and V. A. Najjar. *Biochem. and Biophys. Res. Commun.* 3: 62, 1960.
13. Sidbury, J. B., Jr. and V. A. Najjar. *J. Biol. Chem.* 227: 517, 1957.
14. Sidbury, J. B., Jr., L. L. Rosenberg and V. A. Najjar. *J. Biol. Chem.* 222: 89, 1956.

GENERAL DISCUSSION

Dr. Robert Weed (University of Rochester School of Medicine): Following up Dr. Rothstein's observations that the uptake of Mg^{++} and Mn^{++} by the yeast cell is mediated by an active transport system (Rothstein, A., A. Hayes, D. Jennings and D. Hooper. *J. Gen. Physiol.* 41: 585, 1958), we were interested in studying the human red cell in this regard (Weed, R. I. and A. Rothstein. *J. Gen. Physiol.* 44: 301, 1960). Unlike the yeast cell, however, the entry of manganese into adult red cells appears to occur by a very slow process of passive diffusion, not influenced by the addition of glucose, adenosine or metabolic inhibitors. The intracellular distribution of the manganese which has been taken up by the cell has been studied by separation of the stroma from the hemolysate, revealing that only 5 to 10 per cent is bound to the membrane and that the rest has actually entered the cell. Ultrafiltration experiments on the hemolysate reveal approximately 50 per cent of the intracellular manganese to be present in an ultrafilterable form. In the uptake of manganese by red cells, the rate-limiting factor appears to be passage through the membrane rather than intracellular binding and in this respect differs from Dr. Saltman's findings in studies of the uptake of iron by rat liver.

Dr. Paul Saltman (University of Southern California, Los Angeles): One comment to indicate that our system is clean. We were concerned with Dr. Christensen's point about the cellular localization of the iron. Is it on the outside or is it nonspecifically distributed within the cell? We made radioautographs of liver slices and cells which were cytologically stained (Bass, R. and P. Saltman. *Exptl. Cell Research* 18: 560, 1959). The iron is definitely in the interior of the cell with very little on the surface. The exciting aspect of our kinetic studies of localization indicated that the initial site for iron binding was not in the cytoplasm, but rather in or around the nucleus, suggesting some sort of participation of nucleic acids in the binding process.

But the question I would like to ask you, Dr. Christensen, concerns the absolute amount of amino acid and metal that are moving at the same time. Is there any correlation between them? This would be very significant with respect to your mechanisms.

Dr. Christensen: The amount of amino acid taken up was always substantially larger than that of metal. We went down as low as one millimolar amino acid levels and could not detect very much effect of half millimolar manganese. We do not feel that there is strong evidence here for the view that the manganese ion is the carrier for the amino acid—the original proposition under investigation. It does seem rather likely that perhaps the temporary presence or the movement of some solutes through the plasma membrane facilitates the bridging by certain metals.

Dr. Scheinberg: I would like to raise one point that relates to your observation that the exchange of iron was much quicker, and much more easily measured, than the net flux of iron. You mentioned this with reference to tissues and to cell surfaces. We have had two sets of observations, on what it is fashionable to call the molecular level, which we have never been able to understand but which may be related to this.

Ceruloplasmin has eight copper atoms tightly bound to each molecule. If we add to a ceruloplasmin solution ascorbic acid in sufficient amount to destroy the blue color, and add radioactive copper in a concentration equal to that due to ceruloplasmin and copper, an exchange of ceruloplasmin copper for Cu^{64} occurs. We can detect this experimentally by passing the final solution over ion exchange resins to remove all the free ionic copper. The radioactivity in the effluent ceruloplasmin usually corresponds to four of the eight copper atoms of the protein having been exchanged for Cu^{64}. The exchange occurs, so far as we can tell, instantaneously, and one needs ascorbic acid to do it (Scheinberg, I. H. and A. G. Morell. *J. Clin. Invest.* 36: 1193, 1957). Now, if we took the same amount of ceruloplasmin, at the same pH and ionic strength, added ascorbic acid to it (but not Cu^{64}) and tried to remove the copper from it, it was slow and extremely difficult to do (Morell, A. G. and I. H. Scheinberg. *Science* 127: 588, 1958). The point that has always troubled us is that the instantaneous exchange reaction led us to believe that copper came off the protein and might be precipitated very quickly—yet it does not in the experiments on copper removal. In some way is this analogous to the observations of Dr. Saltman's to which I referred?

Dr. Christensen: Since chelation chemistry seems to be providing an example to help the biologist to understand exchange behavior, I think that it may be worth talking about some phenomena of transport behavior that are probably outside the scope of our present conference.

First of all, in transport we have come to understand that we usually get no measure at all of net migration by measuring the rate at which a labeled solute passes from one phase to another. The chemical intermediate frequently involved in such transport may exchange a solute molecule already taken up for another one from the first phase, very much more readily than it will permit a net migration.

This behavior appears to be highly analogous to that of a chelate which, although very stable to net dissociation, will exchange its metal ion extremely rapidly with a pool of the free metal ion. Nor should we be surprised to find the rate of this exchange related to the concentration of free metal ion. This brings us to a second exchange phenomenon encountered in transport, that of *flow driven by counter flow*. (Rosenberg, T. and W. Wilbrandt. *J. Gen. Physiol.* 41: 289, 1957). In the experiment

of Heinz and Walsh (Heinz, E. and P. M. Walsh. *J. Biol. Chem.* 233: 1488, 1958), the Ehrlich cell is permitted first to accumulate unlabeled glycine. Subsequently, the influx of labeled glycine is found to be directly related to the level of glycine present *within* the cell. This variation in influx produced by varying the internal glycine level is all due to changes in the *exchange* rate, i.e., the presence of glycine on the inside accelerates the exchange. This behavior also seems likely to rest on a simple chemical basis, although it does seem to require some kind of separation or insulation between the site at which the solute is received by the membrane from outside the cell, and the site at which it is discharged into the cell.

DR. SALTMAN: There are two aspects of your question, Dr. Scheinberg, which are important. The answer to our original efflux results are explained by Dr. Christensen's position that because we were at nonphysiological levels of iron, we were just loading all sorts of rather nonspecific iron sites in the system. We have not investigated efflux in our new 1960 model system where we are presenting the iron to the cell in the form of transferrin rather than as ferric ammonium citrate and thus obtain some sort of genuine competition between biologically competing sites.

The other fascinating aspect of your problem with metal-binding proteins is that recently Ben Davis in our laboratory has measured the two binding constants for iron to transferrin. It appears that these two constants differ by a factor of 100. It is quite possible that in this situation you have two different kinds of binding sites for the metal. The affinities are such that under particular chemical environments you would strip only one of the metal ions. For example, the affinity for the first iron going on to apotransferrin is about 10^{29}; the second one, about 10^{27}. This may be the reason why you only find half of the sites on transferrin loaded with iron in the normal physiological situation.

DR. SCHEINBERG: It is very difficult to exchange more than half of the copper atoms in ceruloplasmin. On the other hand, even though it is harder to do, we *can* get all the copper off when we make the apoprotein.

DR. SALTMAN: Did you put in an excess of Cu^{64} as compared with the amount of copper in the protein?

DR. SCHEINBERG: That is right and then we got an exchange that was somewhat greater but we didn't exchange all the ceruloplasmin copper. I thought that it was also implicit in what Dr. Christensen said that one doesn't lose iron from within the cell unless one has iron outside the cell. Is that not true?

DR. SALTMAN: That is correct.

DR. SCHEINBERG: So the observation is still one that is difficult to understand.

DR. SALTMAN: No, I don't think so at all. I think it is still a displacement reaction. It is easier for an iron atom to move on to the binding site and to displace a bound iron than it is, say, for a hydrogen ion.

DR. COTZIAS: Why isn't this called simply diminution in specific activity, as the isotope people say?

DR. CALVIN: I want to take just a few minutes to try and give some kind of a picture of this—why exchange reaction may be faster than dissociation followed by reassociation. This seems to be something which has been tossed around both in terms of the amino acids, and in terms of the copper in and out or the iron in and out, as the case may be. It is certainly true that there are many observations of this kind. In general, exchange reactions for which the initial state and the final state are identical are faster than reactions in which the initial state and the final state are not identical. There seems to be one or two points that one can make which conceivably might account for this in a general way without going into a specific examination of each mechanism.

The energies of the initial state, that includes the bound copper, in this case, and the ceruloplasmin and the hydrogen ion or the hydroxy ion, the water, everything that is involved in this, may be represented here in the initial state. In the final state an exchange reaction, as Dr. Scheinberg points out, is also identical in all its species; that is, there is no change in the energy levels because you start out with bound copper and some free copper and certain hydrogen ions and certain hydroxyl ions, and you finish up with the same amount of bound copper and the same amount of free hydrogen ions and free copper and so on. There is no difference in the energy levels of the two states. There may be an energy barrier of some sort in the transition state in going from the initial to the final state and this will be determined by the detailed mechanism of the reaction. However, if instead of an exchange reaction you first dissociate, you first require dissociation of the copper. Now, what is this? It means that the copper in the case of ceruloplasmin has to be replaced by some other cation, usually hydrogen ion. That would be the normal kind of behavior. There might be other ions involved in it, too. In any case, whatever it is, it is not a displacement by copper ions; it is displacement by something else so there must be a state between beginning and end which is of different energy. In general, if the dissociation goes very rapidly, then, presumably, displacement of copper by hydrogen ion will lead to a state which is lower down and then you will have to go back up to get in the copper. Or, the other way around, the dissociation may be very slow and there may be an energy barrier; then the hydrogen bound system with the free copper must lie above the initial state so there will be a barrier in the beginning. It will fall back down easily enough. However, one will have to get up before one gets down again. So either way, there is, in addition to the ordinary barriers between stable states, this difference—namely, that if the dissociation goes very well, then one goes downhill in the beginning, but if the dissociation goes very slowly then one has to go up hill first and then down hill. (By dissociation I mean replacement of the metal or the amino acid or what have you, by hydrogen ion or hydroxy ion or water molecule.) I think that probably, intrinsically, the exchange reactions, because the final state and the initial state are identical, involve a lower potential barrier—a potential barrier which is less liable to be high than in the other cases.

DR. CHRISTENSEN: The word *transport* has become extremely popular and one can hear it used very freely, for example, for the fixation of a metal by almost anything at all. This usage seems meaningless to me. The definition under which I have used the term *transport* and which I would urge on others is this: that we are discussing the way in which a solute gets from one phase to another, being in the same final state in the two phases. Other processes such as group translocation may have their own interest, but confusion arises when they are termed transports.

DR. MALMSTRÖM: Dr. Scheinberg presented evidence that there are two types of copper in ceruloplasmin, and I would just like to add that we have ESR data to the same effect. The Cu^{++} signal recorded for ceruloplasmin only accounts for 50 per cent of the total copper. This could mean that the other half is Cu^{+}, though it is theoretically possible under some conditions to get a line broadening so that Cu^{++} would not be detected. However, no matter what the cause, the data indicate that half the copper is in a different state than the other half.

DR. HARRY A. SAROFF (National Institutes of Health):

I would like to call attention to the sodium and potassium chelating systems which occur in myosin (Lewis, M. S. and H. A. Saroff. *J. Am. Chem. Soc*, 79: 2112, 1957) and (Saroff, H. A. *Arch. Biochem. Biophys.* 71: 194, 1957). This chelation is similar to that occurring in sodium benzoylacetone (Sidgwick, N. V. and F. M. Brewer. *J. Chem. Soc.* 127: 2379, 1925). Studies on the pH dependence of the binding of sodium and potassium to myosin implicate the amino and imidazole groups as binding sites. We postulate that the chelating site in myosin is made up of the carboxylate ion and the uncharged amino or imidazole group. The log of the binding constants are in the order of 3. This constant is large enough so that biological systems might exhibit important competitive effects between Na^+ and K^+ and other metals.

Dr. Borg: I wish to return to the question raised by Dr. Christensen of exchange produced by loading a system with carrier. I would propose that such an exchange reaction is to be expected in reversible steady-state systems in equilibrium; and I do not see a requirement for an intermediate state of any kind. Take the example of a reversible association reaction of a ligand and its bound metal ion. The rates of dissociation of many sterically unhindered cation complexes are on the time scale of molecular vibrations, that is, in the range below a micro-microsecond. Hence, even with a thermodynamc dissociation constant as small as 10^{-8} or 10^{-9}, which merely defines the statistical distribution of bound and unbound reactants at any given instant, each radioactive tracer atom bound to such a ligand will become briefly dissociated many times per second; and there will be a rapid exchange, or turnover, of bound tracer cations with free ions in the immediate vicinity. However, there will be competition between free tracer ions and free carrier ions for the "rebinding" of the free ligand sites following each dissociation event. Clearly, the dilution of tracer by flooding with carrier—in other words, lowering the *specific activity* of the radiisotope in the free ion pool—will soon decrease the *absolute amount* of bound tracer and will increase the *absolute concentration* of unbound or "free" tracer in the vicinity of the ligand. Since the diffusion or other passive transport of the tracer ions away from the neighborhood of the binding site will depend on the concentration gradient of *tracer* ions, this migration will be enhanced by the loading with carrier. Thus the exchange of bound radioactive metal ions upon challenge with their respective nonradioactive isotopes should be a nonspecific phenomenon applicable to many kinds of systems, such as to cellular sites in intact tissue, ion-exchange resins, equilibrium dialysis, ultrafiltration, etc. So general is the exchange reaction, that there is no onus upon the investigator to justify its presence but rather to explain its absence by means of steric hindrances, transport carrier saturation, the statistical effects of polydentate ligands, or other mechanisms.

Dr. Norman C. Li (Duquesne University, Pittsburgh). We have been working with complexation studies with the uranyl ion, using ion exchange resins. At the pH you are using the uranyl ion probably exists as polynuclear hydroxyl complexes. I would like to ask if you see any difference in the distribution as pH is changed. Also, with regard to the amino acid transport, you mention the binding by the amino acid to the manganese ion but you didn't say anything about the uranyl ion. We have reported recently (Li, N. C., E. Doody and J. M. White. *J. Am. Chem. Soc.* 80: 5901, 1958) that most amino acids do not complex with the uranyl ion because of the very rapid hydrolysis of this ion.

Dr. Christensen: Dr. Weed reminds me that these experiments were made at pH 4, at which pH a good deal of hydrolysis probably had occurred. Uncertainties about the actual activity of a metal ion under study in such experiments are even more serious than has been mentioned when we work at very low levels of the metal ion. Even though our solution is simple to begin with we may very well derive chelators from within the cell, so that the actual initial state of the metal may be not what we think it is.

Dr. Schwarz: What I am going to say seems almost unfair to an audience like this. It concerns the fact that in any biological system, such as ascites tumor cells, the incorporation of an agent from the outside into the cell may not occur by permeation of membranes, but by pinocytosis, i.e., through migration of microscopically or electron-microscopically visible vacuoles into the cell, with subsequent resorption of what was originally "cell wall", and excretion of the undesirable constituents into the medium. Recently, for instance, it was found by electron microscopy that in a system as simple as the incorporation of glucose into fat tissue pinocytosis may be important. There is a terrific acceleration or increase of pinocytosis by insulin. The hormone will enhance this approximately 50- to 100-fold. Under our experimental conditions, glucose uptake by 100 mg of fat tissue amounts to approximately 40 micrograms per hour in the presence of insulin. The concentration of glucose in the medium is such that merely 0.04 cc would have to be incorporated by pinocytosis into 100 mg of tissue within one hour, if this process would be the only way of glucose uptake. We don't really know how much is getting into the cell by this route, but I believe it advisable to ascertain that pinocytosis is not involved in any case in which incorporation of exogenous constituents into cells is studied and discussed. This could be done by electronmicroscopy or other suitable methods. The cell surface is a very complicated and highly organized structure, and it has a lot of life in it, as one can actually see under the microscope.

Dr. Christensen: Pinocytosis as the mode of uptake of small solutes presents some very serious, if not insuperable, problems. In order for the Ehrlich cell to take up by pinocytosis the quantity of glycine actually entering the cell, it would need to introduce into its interior about five times its own volume of extracellular fluid per minute. Then would begin the real transport problem because the cell must then excrete all that water and many of the other molecules. Furthermore, kinetic studies such as those of Heinz completely exclude this kind of a mechanism. Now as for the experiment of Barnett and Ball, what they saw under the electron microscope, a long interval after the actual experiment, was a frequent invaginated appearance of the cell border that was interpreted to indicate that pinocytosis had been accelerated. One does not, of course, see pinocytosis occurring under the electron microscope. Furthermore one may calculate from their results or those of Cahill that, in order to obtain the glucose shown to be taken up, 0.02 ml of intracellular fluid (included in 1 gram of insulin-stimulated adipose tissue) would need to engulf 4 ml. or more of extracellular fluid in an hour to clear it of glucose by pinocytosis. The intracellular fluid would apparently need to receive as much as a thousand times its own volume from the extracellular phase. Their observation is surely an important one. The response of the cell boundary to insulin addition probably is highly relevant to the increased transport, but I question whether vacuole formation represents the principal mode of that transport. Remember that an engulfment of extracellular fluid really leaves the cell with a worse problem of transport, how to get rid of every other molecule but the solute in question, including 55 moles of water per liter.

Dr. Cotzias: These remarks are very timely because Bessis (Bessis, M. and J. Breton-Gorius. *Ann. Recherche Medicale* 2: 151,

1957) believes that pinocytosis might govern a great deal of the iron transport.

DR. SCHWARZ: Dr. Najjar, if I remember correctly, there is a report in the literature by Stickland (Stickland, L. H. *Biochem. J.* 44: 190, 1949) that chromium is particularly effective in activating the phosphoglucomutase reaction. This would be exceedingly interesting in relation to problems on which we have been working. As you may know, we have found that chromium is necessary for maintenance of normal glucose tolerance (Schwarz, K. and W. Mertz. *Arch. Biochem. Biophys.* 85: 292, 1959 and these proceedings, p. 111). It is a dietary agent apparently essential for normal glucose utilization. I wondered whether you had any experience with chromium in activating your crystalline phosphoglucomutase, and whether you know what effect various chelating agents may have on chromium in this system.

DR. NAJJAR: We have not measured the effect of chromium. We have found cobalt and manganese to be inactive. The mutase system seems to be a magnesium activated system.

DR. SCHUBERT: This is a question which concerns Dr. Snell as well as Dr. Najjar. In many of the models showing the effect of the metal on catalysis, bonds are labilized. I am wondering if the products were not known beforehand whether you would be able to determine which bonds are labilized and where the splitting occurs.

DR. NAJJAR: Knowing the products helps a great deal. Because a number of examples of metal-activated systems exist in organic chemistry and enzymology in which bonds are labilized, one can use these in similar instances or project them in related instances.

DR. SALTMAN: Were any other chelating agents active?

DR. NAJJAR: Cysteine is the most active in the system. Histidine and imidazole are active to a slightly lesser extent. Similarly, 8-hydroxyquinoline and EDTA have been found effective. In the preactivation system, we have only worked with imidazole and histidine and both are equally active.

DR. SCHUBERT: Is histidine actually bound to the enzyme or does it influence some other component of the reaction mixture.

DR. NAJJAR: We do not know whether histidine or imidazole are fixed on the enzyme surface during preactivation. This we presently are attempting to determine.

Manganese versus magnesium: why are they so similar in vitro and so different in vivo?

GEORGE C. COTZIAS

Brookhaven National Laboratory, Upton, L. I., New York

In the field of metal chelation, as elsewhere in biology, much of the experience has been gained *in vitro*. The details of such observations often are applied in the construction of models presumed to reflect the body as a whole. The present communication deals with a dichotomy between these two levels of observation and with the reconciliation of this dichotomy. The example thus presented might be of more general meaning since it pertains to the matter of specificity of physiological coordination and chelation.

First thesis of the present communication: A transition element (manganese) may be substituted for extensively *in vitro* by an alkaline earth (magnesium) and *vice versa*. It will be stressed that this substitution of one metal for the other constitutes almost a general rule, independent of the experimental material and of the reactions under investigation.

Second thesis: The intact mammalian organism differentiates sharply between magnesium and manganese. The supportive arguments will be drawn from a brief comparison of the physiological, nutritional and toxicological phenomena as well as isotopic observations involving these metals.

Third thesis: The dichotomy evident between the two investigative levels (*in vitro* vs. *in vivo*) may be reconciled on the basis of the fact that manganese assumes valences higher than $^{+2}$ *in vivo* while magnesium does not. *In vitro* both elements have been studied mostly in the divalent state.

Presentation of these facts seems important because it might lead to a separation of artefactual observations *in vitro* from those having direct physiological bearing. The bulk of the material here has been taken from the literature. Some of the data pertaining to manganese are from the author's laboratory and in part are presented originally here.

I. Limited specificity of in vitro systems

As was stated above, many of the systems studied *in vitro* have shown a metal requirement which could be satisfied either by magnesium or by manganese. This has been a recurrent theme in the present conference and is therefore already heavily documented here.

It should be noted at the outset that in many of the experiments to be discussed use was made of metal additives in quantities which greatly exceed the normal concentrations of these metals in fresh tissues. This fact, where applicable, might be suspected as having brought into play secondary nonspecific tissue sites, following the oversaturation of sites normally involved in metal action.

With regard to enzyme systems *in vitro* which may be activated by either Mg^{++} or Mn^{++}, Table 1 presents a partial list (4, 9, 37). Among them, arginase is noteworthy because it has been studied both *in vitro* and *in vivo*. Mohamed and Greenberg (22) among other investigators have shown that the arginase activity of tissue preparations may be markedly activated following the addition of any of a number of metals. The activation has been most striking in the case of previously dialyzed samples. Its magnitude seemed to have depended upon the kind of metal activator, its concentration, the periods of preincubation and of incubation, the ambient temperature and other such parameters. These experiments show a marked lack of specificity of this enzyme with regard to its metal requirement.

Boyer, Shaw and Phillips (10) as well as Shils and McCollum (28) have found low arginase activity in the livers of manganese-deficient rats as compared to controls receiving adequate manganese supplements in their diets. The latter investigators were able to raise the arginase activity of the deficient livers by means of the addition of manganous salts *in vitro*. Nevertheless, in the context of the present discussion, it would be interesting if this study were to be repeated in order to ascertain whether other metals, the intake of which had not been knowingly restricted by Shils and McCollum, are capable of activating the arginase activity of manganese-deficient animals. Thus, it seems to this author, it will be possible to assess the bearing of these experiments on the matter of specificity as here discussed.

Among the other enzyme systems listed in Table 1, the oxidative phosphorylations are obviously noteworthy. Their activation by either magnesium or manganese has caused some speculation as to the identity of the metal activator *in vivo*. In the hands of Bronk and Kielly (11) manganous additives seemed to have resulted in slightly more pronounced activation than did those of magnesium.

A similar slight preference for manganese as contrasted to magnesium was discovered by Tietz (33) in her studies of a fatty acid synthesizing system. Furthermore, Lindberg and Ernster (21), as well as Zetterström and Ernster (39), have expressed the opinion on the basis of *in vitro* work that manganese (rather than magnesium) might be the necessary cofactor of oxidative phosphorylation. It has to be noted again, however, that the answer to this question must remain moot because of the relatively small differences between the activations produced by these two metals in spite of the abnormally high concentrations necessitated by most of these experiments.

The studies summarized above tend to relate the effects of magnesium and of manganese to mitochondrial *function*. Mitochondrial *structure* has been, however, also related to the activity of these metals. Fonnesu and Davies (17), for instance, have found that either magnesium or manganous salts tend to preserve the structural integrity of isolated, washed liver mitochondria by preventing their swelling.

Even if *all* metal-activated enzymes were nonspecific with regard to their metal requirements, specificity of function could still be exhibited *in vitro* provided that the *transport mechanisms* were of sufficient specificity to discriminate sharply between these metals. Interestingly enough, Rothstein's group (25) has shown a common path of transport for both magnesium and manganese into isolated yeast cells. That this common mechanism requires energy was confirmed in detail by Jennings *et al.* (19). These findings seem of crucial importance in terms of the essentiality of either magnesium or of manganese for cellular life. If neither the mechanism of transport nor the intracellular function of these elements be specific, either one should support life in the absence of the other. In bacteria, Bentley, Snell and Phillips (6) have shown that manganese seems to be essential for *in vitro* growth and have even used this experience in their bacteriological determination of manganese, presumably in the presence of magnesium. No similar study with yeast cells is known to this author. It is obviously important to ascertain whether substitution of magnesium for manganese and *vice versa* can support the life of yeast. If the mechanism which transports these metals into the cells is common for both and if it leads them into an intracellular milieu where their functions are common, either metal should support life in the absence of the other. It will become apparent later why the author seriously doubts that this is the case.

In order to strengthen the argument that the *in vitro* studies show, thus far, mostly identity of behavior of magnesium and of manganese, two additional examples are helpful. First, studies of antibiotic effects on bacteria *in vitro* have shown that either magnesium or manganous ions (2) may protect these cells from the action of some antibiotics. Second, in studies of bacterial mutagenesis (induced by manganous ions) Sarachek (26) has found that magnesium antagonizes the manganese effect. This indicates possibly a common site of chromosomal binding for both elements.

TABLE 1. *Partial List of Enzymes Activated in vitro by Either Mg^{++} or Mn^{++}*

1) Deoxyribonuclease
2) Phosphatases
3) Exopeptidases
4) Dipeptidases
5) Arginase
6) Cysteine desulfhydrase
7) Fatty acid synthesizing systems
8) Ketoacid decarboxylase
9) Enolase
10) Mitochondrial oxidative phosphorylation
11) Carnosinases
12) Prolinases
13) Cholesterol synthesizing systems

It seems to this author that the sum total of the evidence sited above tends to confirm the first stated thesis: various systems *in vitro* tend to be capable of utilizing either magnesium or manganese and are, therefore, not too specific within this limitation.

II. EVIDENCE INDICATING SHARP DISCRIMINATION BETWEEN MAGNESIUM AND MANGANESE WITHIN THE MAMMALIAN BODY

A. Body composition. The adult human body contains roughly 40 mg of magnesium per 100 gm of fat-free tissue (38). In sharp contrast, the best current estimates for manganese would be about 16 μg per 100 gm lean tissue (18, 27). In other words, the ratio of molar concentrations between magnesium and manganese seems to be rougly 2000. This number is rendered even more meaningful since, as will be seen below, the concentration of both elements in tissues seems to be quite steady and this is accomplished by means of separate mechanisms of homeostatic control. It should be noted furthermore that both elements are overwhelmingly intracellular in their distribution, at least as far as cellular organs are concerned.

Bone is among the richest tissues of the body in both magnesium and manganese. Glandular organs are also rich in both elements. A quantitative comparison of the relative distribution of these elements is difficult at the moment because no direct comparative studies in the same organs of the same species are available. In addition, there seems to be some uncertainty as to the values for manganese (13). It is very opportune, therefore, that Thiers and Vallee (31) have studied the intracellular distribution of several elements in fractions of perfused rat liver, particularly since the concentrations of magnesium and of manganese were ascertained concomitantly. As was noted, the analyses were performed on livers previously perfused with sucrose solution. It is very doubtful, however, that this maneuver alone can be held responsible for the very marked differences in intracellular distributions exhibited by these two metals. In addition, the authors have presented convincing control experiments of their own and arguments from the literature supportive of the reliability of their results. Therefore, the relative intracellular distribution of magnesium should be considered as differing markedly from that of manganese. Viewed together with the differences in body composition alluded to above, the findings of Thiers and Vallee (31) support the thesis that magnesium and manganese are sharply differentiated by the body.

B. Absorption and excretion. Experiments with inorganic forms of both of these minerals seem to show that, while the absolute amount absorbed *increases* with the amount in the gut, the fraction absorbed decreases roughly in inverse proportion to the quantity available for absorption in the gastrointestinal tract (3, 13, 14, 34). Neither the loci nor the relative rates of their absorption can be compared at the present, again for lack of comparable data. The absorption of both is affected by many nutritional factors (3, 13, 14, 34, 37) which in some instances, (i.e., excessive calcium) are identical for both elements.

As far as the daily requirements are concerned, Tibbets and Aub (32) have shown that 222 mg of magnesium taken daily resulted in maintenance of metabolic balance in young, normal adults. In sharp contrast, only about 5 mg of manganese are ingested daily by normal adults according to Kent and McCance (20) and Basu and Malakar (5).

With regard to *excretion*, the differences between manganese and magnesium metabolism are striking.

Manganese is excreted almost exclusively in the gastrointestinal tract, to a large extent *via* the bile (7, 13). Therefore, the manganese concentration of the feces reflects not only the amount of metal not absorbed but also that absorbed and excreted. Hence, the gastrointestinal contents constitute both source and sewage for manganese (13). In hitherto unpublished experiments, Cotzias and Selleck have found that homeostasis of manganese (like that of sodium and unlike that of iron) is brought about primarily by the control of the rate of the element's excretion. As far as the biliary route is concerned, Bertinchamps and Cotzias (7, 13) have found that the excretion of injected radiomanganese occurs in two separate "waves" if the animals had been maintained on ample amounts of manganese (50 ppm in the diet or higher) and only in one "wave" if low amounts of the element were ingested (10 ppm or lower). These findings were interpreted as indicating the existence of an accessory metabolic pathway for manganese, which becomes utilized under conditions of manganese loading. The alternate pathway might reflect an alternate valence state of this metal.

The excretion of *magnesium* seems to occur primarily *via* the kidney (1) and fecal magnesium reflects, therefore, primarily the amount of element not absorbed. On the other hand, the urinary loss reflects the amount absorbed and excreted. Fitzgerald and Fourman (16) have studied the obligatory loss of magnesium by the human kidney.

TABLE 2. *Manifestations of Deficiencies*

Magnesium	Manganese
1) Vasodilation	1) Structural and chemical anomalies of bone
2) Purpura	2) Ataxia
3) Erythema Alopecia	3) Female: sterility Male: impotence
4) Excitability	4) Anomalous fat and lipid metabolism
5) Audiogenic convulsions	5) Poor growth
6) Tetany	
7) Visceral degeneration	
8) Metastatic calcification	
9) Poor growth	

TABLE 3. *Toxicity*

Magnesium	Manganese
Acute poisonings	
1) Fall of blood pressure	1) Convulsions
2) Cardiac arrest	2) Low peristalsis
3) Narcosis	3) Shock
Chronic poisonings	
Not known	"Parkinsonism"

They found that upon severe restriction of dietary magnesium, some depletion of body magnesium stores occurred due to this obligatory urinary loss. Barnes, Cope and Harrison (4) fixed the obligatory renal loss at about 1.0 mEq per day and observed almost no fecal loss under conditions of privation. These authors concluded that magnesium can be rigidly conserved by virtue of renal mechanisms. They fixed the maintenance requirement for an adult at about 1 mEq per day.

The above experiments indicate that the body differentiates sharply between the excretion of manganese (fecal) and that of magnesium (renal).

C. Comparison of magnesium and manganese deficiencies. The magnesium deficiency states have been observed both in humans (35) and in animals while those of manganese have been studied extensively in animals only (13, 14, 28, 34). A comparison of their manifestations is valid since in both cases documentation is ample. Table 2 offers such a comparison.

The only striking similarity seems to be the occurrence of poor growth both with magnesium and with manganese deficiency. Poor growth, however, seems to accompany any deficiency and is, therefore, not to be taken as a specific sign. Thus, it merely stresses the fact that both magnesium and manganese are essential nutrients for growth. However, poor growth does not indicate *per se* the mechanisms in which these elements participate.

It is self-evident from Table 2 that the two deficiencies differ sharply in all other essentials. In support of the present argument, it was considered sufficient to show that the limiting steps in the exhibition of deficiency reside in sharply different loci in the case of the one metal than in the case of the other.

D. Manganese versus magnesium poisonings. In order to underline the *in vivo* differences between the metabolism of magnesium and of manganese further, Table 3 was prepared (30, 36). The differences between the acute poisonings are self-evident. They indicate that under conditions of acute metabolic load, a different set of organs seems to be affected in acute magnesium poisoning than in acute manganese poisoning, thus supplementing the illustration offered by the comparison of the deficiencies.

A detailed comparison of chronic poisonings is not possible at the moment since the only clear-cut syndrome is that of chronic manganese poisoning of man (13, 24). A chronic magnesium poisoning might be occurring in some forms of chronic renal disease (29). In spite of the fact that the manifestations of magnesium poisoning, if any, cannot be differentiated from those of the under-

lying disease, it is obvious that the classical form of manganese poisoning (which resembles the Parkinsonian syndrome) is not being duplicated in kidney disease with magnesium retention. Thus, even here, the creation of similar syndromes by the respective chronic poisonings with the two metals seems improbable, at the present.

E. Direct evidence that magnesium does not displace manganese in the body. Cotzias and Greenough (15) have studied the specificity of the manganese pathway through the body, in order to ascertain whether the lack of specificity encountered *in vitro* characterizes also the intact animal organism. They have developed an isotopic technique which permits the direct assay of the displacement of one metal by another within the body. With this technique, the well-known displacement of strontium isotopes by calcium salts was observed, as well as the displacement of Sr^{85} by magnesium salts *in vivo* (Cotzias, G. C., B. Selleck, J. J. Greenough and B. Curtis, unpublished).

Furthermore, the author and his associates have hitherto unpublished studies indicating extensive displacement of parenterally administered Zn^{65} by cadmium salts, regardless whether these were injected or fed.

The experiences with Mn^{54} are, however, in direct contradistinction to those with Sr^{85} or Zn^{65}. The injected Mn^{54} was readily eliminated from the bodies of mice *only* following the administration of stable manganese salts. Indeed, the valence state of the stable element seemed to be immaterial since the elimination of the radioisotope could be brought about by Mn^{0}, Mn^{++}, Mn^{+4} and Mn^{+7}.

In sharp contrast to the effectiveness of the manganese salts in bringing on accelerated elimination of Mn^{54}, metabolic loads with other metal salts failed to affect the normal turnover rate of Mn^{54}. Of immediate pertinence is the fact that, among them, magnesium was as ineffective as control saline solution in affecting the turnover rate of Mn^{54} in the bodies of intact mice. Since the moles of magnesium administered markedly exceeded the total moles of manganese contained in the bodies of these mice, these experiments indicated that the two elements pursue, at least in part, different paths in their passage through the body. This conclusion was rendered particularly plausible by the facts that *1*) radio-manganese was readily eluted out of the bodies of these mice by stable manganese and *2*) that magnesium was not inactive by these criteria and in these animals since it had been seen to accelerate the elimination of Sr^{85}.

The *in vitro* experience presented at the outset is in sharp dichotomy with the *in vivo* experience just discussed on the basis of the recognition of magnesium and of manganese as different elements: the former showed identity while the latter showed exquisite differentiation of these two metals.

III. RECONCILIATION OF THE DICHOTOMY BETWEEN BEHAVIOR OF MAGNESIUM AND OF MANGANESE IN VITRO VERSUS IN VIVO

It was evident to us at the time of the performance of the experiments to be described below that such a reconciliation will have to be brought about by the discovery of at least one gross difference between an *in vitro* and an *in vivo* system rather than by an elaboration of the many differing subtle details between such systems. A gross finding of this nature might reveal an approach to the problem of preserving the role of *in vitro* studies as guides to the study of intact organisms.

It was obvious that one such gross difference between an isolated system (i.e., liver slice) and the intact organ *in situ* (i.e., liver) is the fact that the latter is elaborately perfused by a complex solution, i.e., plasma. A specific step had been postulated as existing in the pathway of manganese through the body and such a step was considered as capable of differentiating manganese from other elements. This step had to be missing *in vitro*. Thus it was obvious that plasma should be investigated for the possibility of its containing carrier mechanisms capable of differentiating between metals and possibly capable of directing the traffic of metals within the body.

As far as *magnesium* is concerned, the work of Copeland and Sunderman (12) and that of Prasad, Flink and Zinneman (23) has shown that binding occurs primarily by plasma albumin, but that alpha-2 globulin also binds some magnesium. It should be noted that albumin and the beta globulins also bind calcium while the alpha-2 globulins do not. These facts might have some bearing or pertinence in explaining the well known effects of magnesium on calcium metabolism and *vice versa*. In addition it is of interest that both plasma calcium and plasma magnesium which are primarily bound to albumin are eventually excreted primarily *via* the kidney, as Bennhold has already pointed out. It would be of interest to speculate whether the small fractions of these metals excreted in the bile might not be the fractions carried by the alpha-2 and beta globulins, respectively.

As far as *manganese* is concerned, a search was made by Bertinchamps and Cotzias (8) for a carrier protein in human plasma. Paper electrophoresis of plasma revealed that the β_1 globulin seemed to bind most of the added $Mn^{54}Cl_2$. Ultrafiltration experiments showed that immediately upon addition of the manganese tracer about 93 per cent became bound. On incubation under oxygen, however, or upon repeated aerobic ultrafiltration of the same sample, this value rose to above 99 per cent. It became evident, therefore, that the manganous tracer might be undergoing an oxidation in the presence of plasma which resulted in its becoming bound to a higher extent on the β_1 globulin. That such must have been the case was confirmed by means of anaerobic incubation (CO_2 or N_2) which interfered with the higher association of Mn^{54} with the binding sites in plasma.

When it became apparent that the firm binding of $Mn^{54}Cl_2$ tracer to β_1 globulin of plasma involved an oxidative step, preliminary experimentation was performed in order to ascertain *1*) whether this binding occurred also *in vivo* and *2*) what the valence state of manganese in the manganese-protein complex might be.

The first question was investigated by means of ultrafiltration of plasma from rats tagged intraperitoneally

with 200 μc of $Mn^{54}Cl_2$ (carrier free). It was found that 79 per cent of the tracer was ultrafilterable initially, while on incubation or on repeated ultrafiltration this value became higher, as had been the case when plasma was ultrafiltered following tagging *in vitro*. Similarly electrophoresis showed that the *in vivo* administered Mn^{54} was primarily bound to the β_1 globulin fraction of plasma.

The matter of the valence state of Mn^{54} in the protein-complex was investigated by means of the use of oxidizing or reducing agents in order to make or to break the complex as judged by ultrafiltration and by electrophoresis. In the presence of buffered CO_2-formate or ascorbate as reducing agents, the complex was observed to become dissociated. Similar observations were made following incubation and H_2. It was shown by separate experimentation that the organic reducing agents decolorized suspensions of MnO_2 without the precipitation of metallic manganese. Thus it is assumed that they stabilized manganese at the valence of $^{+}2$. Therefore Mn^{++} was not the preferred valence state for the formation of the globulin-metal complex.

In experiments in which either H_2O_2 or $K_2S_2O_8$ were used as oxidizing agents, the following results were obtained: when stable $MnCl_2$ or $MnSO_4$ solutions were exposed to these agents, the clear, colorless manganese solutions became khaki-brown in color with the eventual precipitation of brown precipitate. No blue permanganate color was observed. These experiments indicated that the H_2O_2 or $K_2S_2O_8$ additives brought upon the following reaction

$$Mn^{++} \rightarrow Mn^{+3} \rightarrow Mn^{+4}.$$

When the tracer Mn^{54} alone was subjected to full oxidation by these agents and then mixed with plasma, the protein-manganese complex could not be observed indicating that Mn^{+4} was not admitted into the complex. On the other hand if plasma was present, the oxidation took place *in the presence of plasma*, and almost total incorporation of the tracer into the complex was observed Thus it was evident that the preferred valence state for the formation of this complex seemed to lie between $^{+}2$ and $^{+}4$, and was, therefore, probably Mn^{+3}. It is of interest that Mn^{+3} is quite unstable as an aqua ion and can, therefore, not be utilized readily for *in vitro* experimentation in the absence of a stabilizing complexing agent such as the β_1 globulin of plasma.

The obvious difference of the binding sites in plasma for divalent magnesium (albumin, alpha-2) and for trivalent manganese (β_1) is sufficient to explain the separate pathways pursued by these two elements *in vivo*. It was of interest to see, however, whether the β_1 globulin was capable of differentiating between these two elements as well. In preliminary competition experiments, it was found that 0.2 M magnesium would displace little, if any, of the Mn^{54} tracer bound to the β_1 globulin. Thus it became evident that Mg^{++} might compete with Mn^{++} but not with Mn^{+3}.

Since the β_1 globulin fraction of plasma also carried plasma iron, it was of interest to determine whether transferrin and "transmanganin" constitute different proteins or merely different systems. Although important differences between the two have been observed, this question remains moot until a clear-cut separation of two different proteins may be achieved. This question is not pertinent to the issue of differentiation between magnesium and manganese in the body.

SUMMARY AND CONCLUSIONS

Evidence was presented which was interpreted as indicating that *in vitro* magnesium may be substituted for by manganese and *vice versa* as a general rule.

Evidence was drawn from chemical, nutritional, toxicological and kinetic observations *in vivo* which was interpreted as indicating that magnesium and manganese are clearly differentiated within the bodies of intact animals and behave metabolically as sharply dissimilar agents.

The relative lack of specificity of the *in vitro* systems was reconciled with the high specificity observed *in vivo* by a comparison of the valence state of the two metals during transport by plasma. It was pointed out that in addition to the separate normal binding sites, a large fraction of plasma manganese occurs in the trivalent form while plasma magnesium is, of course, divalent. The necessary redox systems which result in the stabilization by binding of Mn^{+3} are sufficient to account for the *in vivo* specificity. If applied *in vitro*, they might serve to differentiate physiological from artefactual phenomena.

REFERENCES

1. Aikawa, J. K., G. S. Gordon and B. L. Rhoades. *J. Appl. Physiol.* 15: 503, 1960.
2. Albert, A. and C. W. Rees. *Nature, London*, 177: 433, 1956.
3. Barker, E. S. *J. Chron. Dis.* 11: 278, 1960.
4. Barnes, B. A., O. Cope and T. Harrison. *J. Clin. Invest.* 37: 431, 1958.
5. Basu, K. P. and M. C. Malakar. *J. Indian Chem. Soc.* 17: 317, 1940.
6. Bentley, O. G., E. E. Snell and P. H. Phillips. *J. Biol. Chem.* 170: 343, 1947.
7. Bertinchamps, A. J. and G. C. Cotzias. *Fed. Proc.* 17: 428, 1958.
8. Bertinchamps, A. J. and G. C. Cotzias. *J. Clin. Invest.* 39: 979, 1960.
9. Boyer, P. D., H. Lardy and K. Myrbäck (editors). *The Enzymes* (2nd ed.). New York: Acad. Press 1959.
10. Boyer, P. D., J. H. Shaw and P. H. Phillips. *J. Biol. Chem.* 143: 417, 1942.
11. Bronk, J. R. and W. W. Kielly. *Biochim. et biophys. acta* 24: 440, 1957.
12. Copeland, B. E. and F. W. Sunderman. *J. Biol. Chem.* 197: 331, 1952.
13. Cotzias, G. C. *Physiol. Rev.* 38: 503, 1958.
14. Cotzias, G. C. In: *Mineral Metabolism: An Advanced Treatise*

edited by C. L. Comar and F. Bronner. New York: Acad. Press, vol. 2, in press.
15. Cotzias, G. C. and J. J. Greenough. *J. Clin. Invest.* 37: 1298, 1958.
16. Fitzgerald, M. G. and P. Fourman. *Clin. Sc.* 15: 635, 1956.
17. Fonnesu, A. and R. E. Davies. *Biochem. J.* 64: 769, 1956.
18. Fore, H. and R. A. Morton. *Biochem. J.* 51: 600, 1952.
19. Jennings, D. H., D. C. Hooper and A. Rothstein. *J. Gen. Physiol.* 41: 1019, 1958.
20. Kent, N. L. and R. A. McCance. *Biochem. J.* 35: 877, 1941.
21. Lindberg, O. and L. Ernster. *Nature, London* 173: 1038, 1954.
22. Mohamed, M. S. and D. M. Greenberg. *Arch. Biochem.* 8: 349, 1945.
23. Prasad, A. S., E. B. Flink and H. H. Zinneman. *J. Lab. & Clin. Med.* 54: 357, 1959.
24. Rodier, J. *Brit. J. Indust. Med.* 12: 21, 1955.
25. Rothstein, A., A. Hayes, H. Jennings and D. C. Hooper. *J. Gen. Physiol.* 41: 585, 1958.
26. Sarachek, A. *Biochim. et biophys. acta* 33: 227, 1959.
27. Schroeder, H. A. *Advances Int. Med.* 8: 254, 1956.
28. Shils, M. E. and E. V. McCollum. *J. Nutrition* 26: 1, 1943.
29. Smith, W. O. and J. F. Hammersten. *A. M. A. Arch. Int. Med.* 102: 5, 1959.
30. Sollmann, T. H. *A Manual of Pharmacology.* Philadelphia: Saunders, 1957.
31. Thiers, R. E. and B. L. Vallee. *J. Biol. Chem.* 226: 911, 1957.
32. Tibbets, D. M. and J. C. Aub. *J. Clin. Invest.* 16: 491, 1937.
33. Tietz, A. *Biochim. et biophys. acta* 25: 303, 1957.
34. Underwood, E. J. *Trace Elements in Human and Animal Nutrition.* New York: Acad. Press, 1956.
35. Vallee, B. L., E. C. Wacker and D. D. Ulmer. *New England J. Med.* 262: 155, 1960.
36. Von Oettingen, W. F. *Physiol. Rev.* 15: 175, 1935.
37. Wacker, E. C. and B. L. Vallee. *New England J. Med.* 259: 431, 475, 1958.
38. Widdowson, E. M., R. A. McCance and C. M. Sperry. *Clin. Sc.* 10: 113, 1951.
39. Zetterström, R. and L. Ernster. *Nature, London* 178: 1335, 1956.

DISCUSSION

Manganese versus magnesium: why are they so similar in vitro and so different in vivo?

I. HERBERT SCHEINBERG

Department of Medicine, Albert Einstein College of Medicine, and Bronx Municipal Hospital Center, New York City

Some of the data which Dr. Cotzias presented in his paper are shown here in tabular form.

	Daily Intake	Body Content/70 kg Lean Tissue	Deficiency States Exist?	Toxicity Exists? Acute?	Chronic?
Magnesium	222 mg	28 gm	Yes	Yes	Unknown
Manganese	5 mg	11 mg	Yes	Yes	Yes

We may note, first, that these data lend further support to Dr. Cotzias' thesis that manganese and magnesium are distinguished sharply by mammals. For if magnesium *could* act like manganese *in vivo*, the large intake of magnesium, relative to that of manganese, might be expected to produce symptoms of manganese toxicity.

Second, it appears that there is less than 1 per cent of the body's content of magnesium in the daily diet. In contrast, the dietary content of manganese is almost 50 per cent of the body's content. Considering these two proportions, we might expect that the organism could have difficulty in supplying its needs for magnesium so that deficiency might develop not uncommonly. Manganese deficiency, on the other hand, is unlikely to occur since in two days enough manganese is ingested in the diet to supply the body's entire requirement. Although deficiency of both metals can, in fact, occur occasionally, chronic magnesium poisoning is unknown while chronic manganese toxicity is well recognized. This contrast may, therefore, reflect the amounts of each element in the diet relative to body requirements of them.

Third, the uncommon occurrence of chronic manganese toxicity in the face of the large dietary intake of the metal relative to body content suggests that there is a specific mechanism which regulates the amount of dietary manganese which the organism retains. Otherwise, perhaps, manganism ought to develop in all of us.

Finally, the possibility that there is a mechanism regulating absorption and/or excretion of manganese suggests, in turn, that similar mechanisms may operate with respect to other metals.

DISCUSSION

Chemical and physiological specificity in metal-binding: A model metal-phenothiazine system involving a free-radical component[1]

DONALD C. BORG

Brookhaven National Laboratory, Upton, L. I., New York

I. CONTRAST BETWEEN PHYSIOLOGICAL AND CHEMICAL EXPERIENCES WITH SPECIFICITY IN METAL-BINDING

In living systems different metal ions may exhibit marked disparities in total content, relative distribution, and subcellular or overall turnover rates. It is tautological that in the final analysis the singularity of a metal's metabolic character must derive from the same chemical properties that determine its general and coordination chemistry. However, there is often a sharp discrepancy between metal ion specificity known *in vitro* and that found in the intact animal (10). In his presentation at this conference Cotzias has documented this for a representative alkaline earth and transition group element (9).

Review of the chemistry of chelation (1, 27, 42) does not explain the exquisite specificity of some metal metabolic pathways (11). Although many metalloenzymes and metalloproteins are known wherein only one or two metals are implicated (13, 28e, 48), the reasons for such biological specificity remain largely obscure (45), although the subject is one of keen current interest (14, 15, 48). For example, all known human metalloprotoporphyrins contain iron (24)—with the probable exception of a trace of manganese porphyrin suggested by recent data (3). Despite this, other metals are readily synthesized into protoporphyrin nonenzymically (2, 24, 40) and even by extracted enzyme systems *in vitro* (R. F. Labbe, personal communication); and they are recognized components of metalloporphyrin-proteins elsewhere in nature (13,15,24). Possibly the virtually absolute specificity for the actual insertion of iron into human porphyrins *in vivo* is but another manifestation of those same ligand properties that presently are envisioned (14, 15, 48) as determining the chemical state of the iron after its synthesis into such molecules has been completed. Conversely, the known existence of other metal porphyrins may imply that the predominate synthesis of iron-containing porphyrins only reflects the physiological complexities that determine the relative availability of metal ions at the cellular site of synthesis. Alternatively, the selective incorporation of iron may indicate an aspect of coordination chemistry not yet fully recognized.

Vallee and his colleagues (8,43,44) have demonstrated that the nature of the metal component required for activity of carboxypeptidases manifests a relatively low order of specificity amongst several transition group cations. Although there is an optimal binding affinity, *per se*, for zinc; cobalt and nickel can serve more efficaciously as enzyme cofactors, and manganese and iron can be active (8). This comparatively unselective response to metals is also representative of other, more labile, enzyme-metal complexes and metal-coordination compounds generally; because in these loosely bound species discrete metal specificity is extraordinarily rare, and a graded spectrum of response characterized by the Irving-Williams or Bjerrum electronegativity series of metal cations (1, 23, 27, 28a, 42, 45, 46) is the rule. Exceptions to this behavior usually are explained by the mutual satisfaction of requirements due to ligand configuration and the special directional orientations of particular metal bonding orbitals (14, 23, 25, 28d, 45, 48), a particular *d*-shell electron spin state induced by ligand fields (20, 25, 37, 38, 45, 48), a suitable valence or redox potential (1, 9, 14, 35, 46, 48) or a restriction of bonding to metals of a certain ionic radius due to local steric factors imposed by the ligand (1, 27, 28b and c, 35, 37 38, 45). No single one of these parameters can by itself distinguish uniquely a particular metal ion; however, it is virtually impossible for two different metals to possess simultaneously the same valence and bonding-electron configuration. Hence a reaction situation capable of impressing sharply restricted requirements with regard to more than one of these parameters could possibly induce a high order of selectivity in metal-binding or function (9). None the less, metal specificity—although amplified—is seldom complete, a generalization that is particularly valid *in vitro* for alkaline earth cations and for the less electronegative members of the first transition series of elements (1, 14, 27, 28c, 45).

In contrast to this, there are physiological studies of

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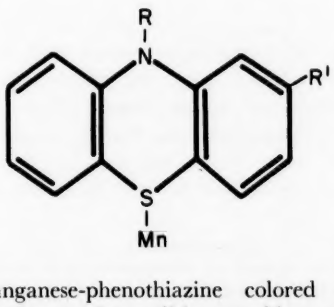
metal metabolism by Rothstein (21, 41), Christensen (7, 36), Cotzias (9–11) and others that depict a remarkably selective behavior for metal ions of generally low specificity *in vitro*, such as magnesium and manganese. Complex dynamic interactions of intricately interdigitating systems, no one of which displays absolute specificity by itself, may occasionally give rise to an overall physiological pathway that is virtually specific for a given metal (14, 46), but no real example of this is known in detail. The physiological and metabolic data, therefore, may imply the existence of an aspect of metal-coordination chemistry that is presently elusive but which can provide extreme specificity of metal interaction in certain instances—perhaps even in simple systems.

II. A SIMPLE MODEL SYSTEM WITH SHARPLY RESTRICTED REACTIVITY TOWARD METAL IONS

We have had experience with an example of what is apparently just such a model system. Phenothiazines are small molecules (31), many of which have widespread pharmacological effects (6, 31) and/or are dyes with intriguing redox properties (31, 39). Chlorpromazine, an important phenothiazine drug derivative, can alter the binding of manganese ions to tissue homogenates and to other proteins (4). Stoichiometry of an apparent manganese-chlorpromazine complex or reaction intermediate has been inferred from several kinds of data. Equilibrium binding of radiomanganese to proteins as affected by the concentration of chlorpromazine, acting competitively (4); structure-reactivity experiments involving measurement of a colored compound formed with manganese cations and a series of substituted phenothiazine derivatives (5); analyses of these chromogenic reactions by the method of continuous variation of Job (5); and studies of the antagonistic effects of known chelating agents on the competitive binding and color-forming experimental systems have been correlated. The stoichiometry concordant with all these data indicates the formation of a manganese-phenothiazine reactive species wherein the heterocyclic sulfur (thioether) serves as the ligand or reaction site on the phenothiazine (5), thus:



The manganese-phenothiazine colored complexes form only under certain conditions; and however formed, they are metastable and photosensitive, bleaching spontaneously over several hours in the dark and almost instantaneously upon exposure to intense ultraviolet light. When trivalent manganic ion is prepared as the brown oxide, MnO(OH), by aerobic precipitation of manganous salt solution with alkali, it can then be added anaerobically to slightly acid phenothiazine solutions; and the characteristic color will then appear as the sparingly soluble Mn^{+3} slowly dissolves. However, if divalent manganese is added to soluble phenothiazine cations in slightly acid solution, no reaction is apparent; and the mixture must be titrated aerobically with base until the positive charge on the phenothiazine is neutralized. Then back titration with mineral acid to the starting pH clarifies the solution and generates the same chromogen seen after reaction with Mn^{+3}. Apparently phenothiazines combine specifically with trivalent manganese, but when charge on the protonated dye is neutralized and its basicity enhanced, Mn^{++} may approach it and then be oxidized in the presence of dissolved oxygen and subsequently react in the trivalent form.

Ferric salts spontaneously form colored products with phenothiazine derivatives within a few seconds after mixing, except that Fe^{+3} more dilute than about one millimolar fails to react, despite the fact that with dilute reagents the manganese reacts as expected. With any given phenothiazine derivative, manganese and iron form chromogens that are spectrophotometrically identical; and in the case of chlorpromazine the spectrum is also identical with the red reaction product previously reported for Fe^{+3}-chlorpromazine by others (16) (see Fig. 1). Fe^{+3} compounds are also metastable and photosensitive, but less so than the corresponding Mn^{+3} species. However, both colored products are quenched stoichiometrically by strong chelating agents and are bleached instantaneously by ascorbic acid or Fe^{++}, while remaining unaffected by other metal salts tested.

In general thioethers are relatively weak ligand sites, and on the basis of "classical" metal-coordination chemistry they would be expected to show significantly greater affinity for the more electronegative transition elements than for manganese and iron (23, 28a, 45, 46, 48). Accordingly, the salient feature of the metal-phenothiazine reaction system is its striking specificity: of all divalent metal ions tested, only manganese is effective, even when the titration-back titration maneuver is employed; and trivalent cations other than manganese or iron are similarly inert (Table 1). Such unique specificity of metal reactivity in a simple chemical system recommends it as a model for instances of discrete metal selectivity seen in living cells or intact organisms but not yet understood, as in the examples cited by Christensen (7) and Cotzias (9). The underlying mechanism of the phenothiazine reaction might also be analogous to that exhibited by complex biological macromolecules which bind only one or two target elements, such as transferrin and the manganese-carrying β globulin of plasma (which may also be a transferrin), discussed by Cotzias (9). Thus determination of the chemical nature

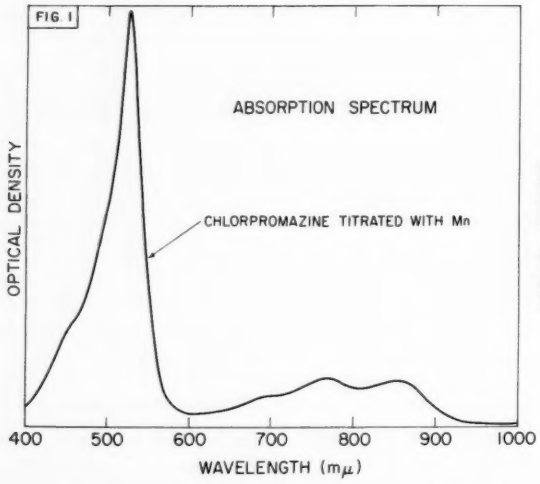


FIG. 1. Spectrum of the manganese-chlorpromazine reaction product in the visible region. The ferric-chlorpromazine compound is qualitatively identical, suggesting that the spectrum is that of the phenothiazine ion radical itself, because this is the component which is common to both the iron and manganese reactions. Complexes with other phenothiazine derivatives show slight (10–20 mμ) shifts in the main absorption band, depending on the substituent groups bound directly to the aromatic nucleus.

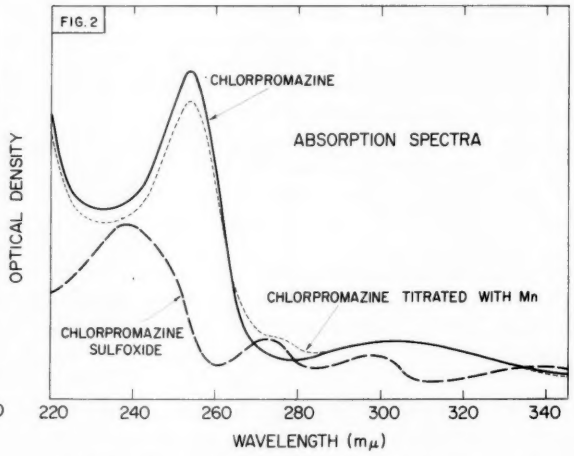


FIG. 2. Spectra of chlorpromazine, its sulfoxide and its manganese reaction product in the ultraviolet region. The ferric complex is qualitatively the same.

TABLE 1. *Test for Formation of Colored Products from Metal-Phenothiazine* Interactions*

	No Color With or Without Titrations	Color Only After Titrations	Color Without Titrations
Divalent	Fe, Co, Ni, Cu, Zn, Mg, Ca	Mn	—
Trivalent	Al, Cr	—	Mn, Fe†

* All metals were tested with chlorpromazine and most were also tested with mepazine and trifluoperazine. † Subsequently other oxidizing metal ions have been found to be effective (Borg. D. C. and G. C. Cotzias: submitted for publication).

of the manganese (-iron)-phenothiazine reaction appears to be strongly warranted.

The appearance of a deeply colored reaction product that is metastable and photosensitive suggests the presence of a "charge-transfer complex" involving an interaction between an electron donor and acceptor that is stabilized by resonance between no-bond and dative structures, as originally defined by Mulliken (35). The manganese (-iron)-phenothiazine system possesses additional features compatible with a charge-transfer process, because the metal ion has already been shown to react only when in the trivalent state, hence potentiating its electron-acceptor properties. At the same time, LCAO molecular orbital calculations made by the Pullmans (39) have suggested that phenothiazines should possess outstanding electron-donor properties, with their highest occupied molecular electronic orbitals being antibonding in character. This argument has been extended specifically to chlorpromazine, for which the possibility of charge transfer as a general mechanism of pharmacological action has been postulated (22).

A special class of intense electronic absorption spectra appear that are characteristic of molecular charge-transfer compounds themselves but derive from neither the donor nor acceptor components alone; and often there is an especially prominent absorption band (the "charge-transfer band") in the near ultraviolet, in addition to any absorption peaks in the visible range (which, by themselves, may be difficult to distinguish from those due to intra-atomic transitions) (32,35). Figure 2 demonstrates that the aromatic phenothiazine ring of chlorpromazine possesses a very strong absorption band at 254 mμ. However, after the titration reaction with manganese, a new band appears at 274 mμ which can be distinguished clearly from the spectrum of chlorpromazine oxidized completely to the sulfoxide state. The 274 mμ peak is followed more readily on the difference spectrum of Figure 3, where it is seen to decay in parallel with the absorption peak at 523 mμ that gives the complex most of its color (see also Fig. 1).[2] Exposure to ultraviolet produces bleaching at both 274 mμ and in the visible range; and preliminary studies with interference-type band-pass filters suggest that u.v. inactivation is most efficient at ~270 mμ, which is to be expected if energy absorption in the "charge-transfer band" promotes the "resonating" electron to almost complete localization about the acceptor (22, 35). Excess u.v. exposure far above and beyond bleaching produces a

[2] The molar extinction coefficient, ϵ, of the 274 mμ peak has not been calculated because the fractional yield of manganese-chlorpromazine compound after the back titration procedure is not yet known, although it is clearly much less than unity. However, a lower limit of $\epsilon \geq 5 \times 10^3$ can be determined by *assuming* the complexing reaction to be 100% efficient. An ϵ of this value is compatible with the range expected for charge-transfer bands (35, 48).

new u.v. absorption spectrum increasingly like that of the sulfoxide.

Further information regarding the nature of the metal-phenothiazine reaction is provided by the finding of a "free-radical type" electron spin signal on electron spin resonance (ESR) spectrometry of the colored compounds (Fig. 4). This is compatible with the transfer of an electron from the phenothiazine donor to the metal-ion acceptor, leaving a "hole" or unpaired electron behind. If the reaction product is a "charge-transfer complex" (35, 48), the donated electron would be promoted only to a new molecular orbital that overlaps both metal (predominantly) and phenothiazine (to a lesser extent in order to explain the ESR signal), rather than being transferred completely to the acceptor. In any case, the lack of hyperfine structure in the free-electron signal could reflect a delocalized orbital for that electron, but more likely it indicates that the unpaired electron is the remaining one of the non-bonding lone pair of electrons originally about the heterocyclic sulfur after the other of the pair has been donated to the charge-transfer or "redox" process. This is consistent with the previous conclusion that the thioether provides the ligand or active site on the phenothiazine (5); whereas the absence of a nuclear magnetic moment in sulfur would explain the simple ESR signal, without fine structure.

The demonstration of a free radical phenothiazine ion confirms the conclusions of Michaelis, Granick, and Schubert of some years ago (18, 19, 33, 34). They proposed that on oxidation in a very acid environment phenothiazines and related oxazines and selenazines would form resonance-stabilized semiquinone radicals. Their absorption spectra of somewhat differently substituted phenothiazines (18) show great similarity to Figure 3, with intense bands at ~280 mμ and at 500-600 mμ. Presumably, the resonance stabilization provided by the proton in the strong acid medium of their experiments is furnished in the present case under more nearly physiological conditions by the metal cation.

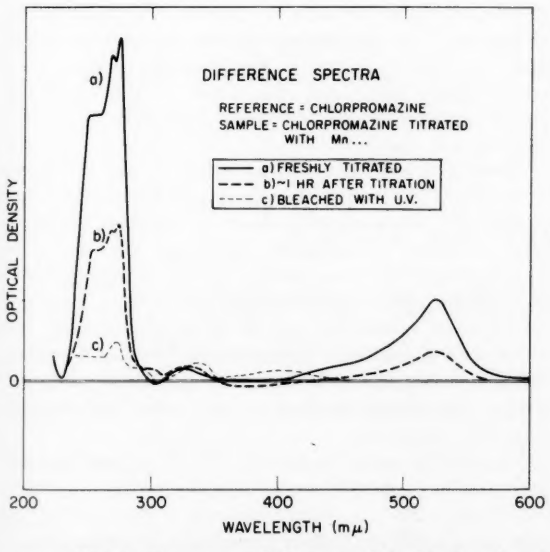


FIG. 3. The band at 274 mμ fades in parallel with the color; *c*) may depict a slight excess of u.v. bleaching with some secondary increase in the peaks at 270 mμ and 330 mμ. With more u.v. these increase significantly to resemble—but not to match—the difference spectrum shown by the sulfoxide.

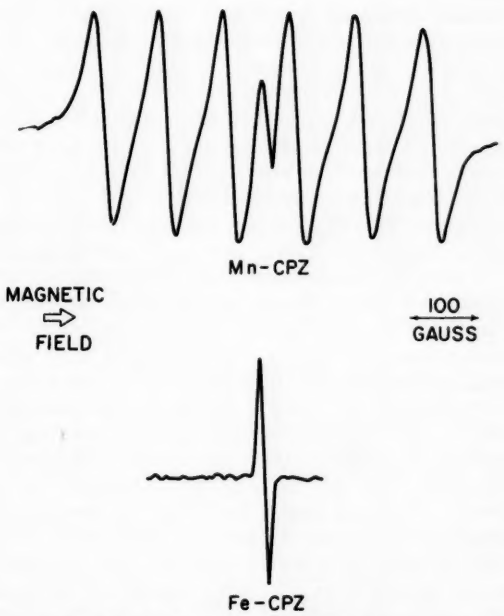


FIG. 4. The "free-radical" signal without fine structure is seen in the ferric-chlorpromazine system, with a "g" value corresponding to that for the free electron. As usual, iron provides no ESR signal. With the manganese reaction the free electron spin signal is superimposed on the characteristic manganese hyperfine structure.

The exact congruity of the absorption spectra of the manganese and ferric iron colored products (Figs. 1 and 3), as well as their similarity to those of the phenothiazine semiquinone radicals (18) discussed just above, would not be expected if the spectra largely reflected charge-transfer bands. Alternatively, the spectra may be those of free phenothiazine radical ions in solution, as formed by the one-electron transfers to higher oxidation states of metal cations proposed earlier. The specificity of the metal reaction then would derive from the need to match the oxidation potential of the metal "redox" couple with that of the phenothiazine oxidation step. The selectivity of such a requirement (28, 46, 48) is emphasized by Table 1: not only do most of the ions listed fail to produce the free radical by themselves, but even when they are mixed with manganese in the reaction mixture, they do not interfere with the manganese-phenothiazine interaction (—the exception being Fe^{++}). In other words, from a mixture of metal cations, the phenothiazine "selects" only those with appropriate oxidation potential.

Even in the presence of phenothiazine radical ions, there may be further coordination with cation to form

a metal-free radical complex. This would provide the resonance stabilization proposed by Michaelis *et al.* (18, 19, 33, 34) and would conform to certain data obtained with chelating agents (D. C. Borg, unpublished). At the present time neither a metal-free radical nor even a charge-transfer complex can be ruled categorically in or out; but investigations designed to clarify this issue are being pursued actively.

However, identification of the metal-phenothiazine reaction as a charge-transfer process also would produce useful insight into the marked metal specificity of the complex. For charge-transfer to take place, the acceptor (metal ion) must possess an electronic structure such that the energy difference between a filled and unfilled orbital falls within the range defined by the limits of the two highest filled levels in the donor, with the binding energy of the complex offsetting any difference between the filled and unfilled orbitals in the donor and acceptor, respectively (29, 30).[3] Thus, there must be overlap between filled and unfilled orbitals in this way for charge-transfer to occur (30). Since the electronic orbital states of atoms of different elements are individually distinct, specificity in the charge-transfer process would derive from an "energy-match" between donor and acceptor levels. If the orbital spacing of the donor is narrow, the energy levels in the two components (donor molecule and acceptor metal atom) must be closely matched, thus resulting in very high specificity for the metal (29).

Although charge-transfer reactions have been proposed in biology, no example of a metal complex stabilized solely by charge-transfer forces has been confirmed as yet (29, 32, 35); although Williams has shown iron-porphyrin and copper-sulfur compounds to possess charge-transfer *spectra*, as such (45, 47). However, Dunn has concluded that charge-transfer is the dominant feature in the binding of trivalent metal ions to ligands generally (12), and there are suggestions that partial electron transfers give rise to some of the optical absorption bands seen in metal-porphyrins (46). Moreover, in his original presentation of the concept, Mulliken (35) proposed that ". . . charge-transfer forces . . . may afford new possibilities for understanding intermolecular interactions in biological systems." At this conference Malmström presented data (26) that during the reaction of xanthine oxidase molybdenum changes valency, and flavin adenine dinucleotide (FAD) free radicals are formed. These events are entirely analogous to those occurring during the formation of the metal-phenothiazine free radical. Possibly the behavior of the free electron as Malmström has observed it in Cu^{++} containing oxidative enzymes (26) is derived from charge-transfer processes.

Because of the small size and simplicity of its ligand, which is susceptible to quite rigorous chemical analysis (22, 39), the phenothiazine model can serve well as an analogue to develop understanding of similar processes in enzymes and other biological macromolecules, whose primary and secondary structures are unknown in all but a handful of instances. It can be hoped that analysis of this model system will prove applicable to some of the otherwise paradoxically-specific metal pathways recognized *in vivo* (9, 11). In any case, the further study of physiological and metabolic data will undoubtedly lead the biologist interested in metal-binding *in situ* or concerned with the synthesis of more effective complexing agents to look more frequently to "quantum phenomena" for new understanding.

[3] In other words, for charge-transfer to take place, the binding energy of the complex must roughly offset the difference between the ionization potential of the donor and the electron affinity of the acceptor (29).

III. A DIFFERENT PERSPECTIVE ON THE ROLES OF TRACE METALS IN BIOLOGY

All too often the biochemist has regarded the metal requirements or sensitivities exhibited by so many enzymic systems as simply other environmental cofactor requirements to be satisfied empirically. Infrequently has the mechanism of metal-binding been examined for the purpose of understanding the macromolecular system with which it is involved (43). Considering the ubiquitous distribution of metal-dependent reactions and molecular structural components, it appears that denominators common to much of biological chemistry are being overlooked.

By virtue of their very simplicity, the chemistry of metal ions can be understood with much greater rigor than can that of complex biological molecules whose detailed structure is rarely known. Papers given at this conference emphasize further that theoretical metal chemistry already is well developed and that further rapid progress is imminent in reducing many properties of metals to the underlying electron "quantum" chemistry (15, 26, 27, 38, 48). The example of our model system has emphasized that some of the distinctive features of metal chemistry and physiology are best explained at this level.

These opinions suggest that there should be a shift in emphasis from regarding metal cofactors as incidental aspects of biochemical systems to viewing them as critical centers of advanced knowledge that can serve as keys to unlock many currently locked doors in biology. Rather than subordinating the role of metals to the present vague concepts of molecular biology, the theories of molecular behavior should be made consistent with or even extrapolated from the more rigorously understood metal chemistry, as has been emphasized in detail by Vallee (43).

Granick has even suggested (17) that most metalloenzymes and metal-enzyme complexes may be traced phylogenetically to their mineral origin. Thus, the chemistry of life may often turn out to be fundamentally the chemistry of the inorganic metal ion, amplified immensely in specificity and efficiency by the macromolecular organic "cofactor" components. Were this rather extreme speculation to bear up, then metal

chemistry would be the body of much of the understanding of living processes, wagging the tail of biological specificity and modification, rather than the converse that is assumed customarily!

REFERENCES

1. ALBERT, A. This symposium.
2. BASES, R., S. S. BRODIE AND S. RUBENFELD. *Cancer* 11: 259, 1958.
3. BORG, D. C. AND G. C. COTZIAS. *Nature, London* 182: 1667, 1958.
4. BORG, D. C. AND G. C. COTZIAS. *Fed. Proc.* 17: 430, 1958.
5. BORG, D. C. AND G. C. COTZIAS. *Fed. Proc.* 19: 248, 1960.
6. BORG, D. C. AND G. C. COTZIAS. *BNL*-4560, 1960.
7. CHRISTENSEN, H. N. This symposium.
8. COLEMAN, J. B. AND B. L. VALLEE. *J. Biol. Chem.* 235: 390, 1960.
9. COTZIAS, G. C. This symposium.
10. COTZIAS, G. C., D. C. BORG AND A. J. BERTINCHAMPS. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 50.
11. COTZIAS, G. C. AND J. J. GREENOUGH. *J. Clin. Invest.* 37: 1298, 1958.
12. DUNN, T. M. *J. Chem. Soc.* p. 623, 1959.
13. EICHHORN, G. L. In: *Chemistry of the Coordination Compounds*, edited by J. C. Bailar, Jr. New York: Reinhold, 1956, p. 698.
14. EICHHORN, G. L. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 19.
15. EICHHORN, G. L. This symposium.
16. FELS, I. G. AND M. KAUFMAN. *Nature, London* 183: 1392, 1959.
17. GRANICK, S. *Ann. New York Acad. Sc.* 69: 292, 1957.
18. GRANICK, S. AND L. MICHAELIS. *J. Am. Chem. Soc.* 69: 2983, 1947.
19. GRANICK, S., L. MICHAELIS AND M. P. SCHUBERT. *J. Am. Chem. Soc.* 62: 1802, 1940.
20. GRIFFITH, J. S. AND L. E. ORGEL. *Quart. Rev. (London)* 11: 381, 1957.
21. JENNINGS, D. H., D. C. HOOPER AND A. ROTHSTEIN. *J. Gen. Physiol.* 41: 1019, 1958.
22. KARREMAN, G., I. ISENBERG AND A. SZENT-GYÖRGYI. *Science* 130: 1191, 1959.
23. KELLER, R. N. AND R. W. PARRY. In: *Chemistry of the Coordination Compounds*, edited by J. C. Bailar, Jr. New York: Reinhold, 1956, p. 174.
24. LEMBERG, R. AND J. W. LEGGE. *Hematin Compounds and Bile Pigments*. New York: Interscience, 1949.
25. LINNETT, J. W. *Discussions Faraday Soc.* No. 26, p. 7, 1958.
26. MALMSTRÖM, B. G. This symposium.
27. MARTELL, A. E. This symposium.
28. MARTELL, A. E. AND M. CALVIN. *Chemistry of the Metal Chelate Compounds*. New York: Prentice-Hall, Inc., 1952, a) p. 167, b) p. 175, c) p. 181, d) p. 238, e) p. 359.
29. MASON, R. *Discussions Faraday Soc.* No. 27, p. 129, 1959.
30. MASON, R. In: *Symposium on Bioenergetics*. Brookhaven Nat. Lab. In press.
31. MASSIE, S. P. *Chem. Rev.* 54: 797, 1954.
32. MCCLURE, D. S. In: *Symposium on Bioenergetics*. Brookhaven Nat. Lab. In press.
33. MICHAELIS, L., S. GRANICK AND M. P. SCHUBERT. *J. Am. Chem. Soc.* 63: 351, 1941.
34. MICHAELIS, L., M. P. SCHUBERT AND S. GRANICK. *J. Am. Chem. Soc.* 62: 204, 1940.
35. MULLIKEN, R. S. *J. Am. Chem. Soc.* 74: 811, 1952.
36. PAL, P. R. AND H. N. CHRISTENSEN. *J. Biol. Chem.* 234: 613, 1959.
37. PEARSON, R. G. *J. Phys. Chem.* 63: 321, 1959.
38. PEARSON, R. G. This symposium.
39. PULLMAN, B. AND A. PULLMAN. *Biochim. et biophys. acta* 35: 535, 1959.
40. RACKOW, B. *Z. Naturforsch.* 15a: 129, 1960.
41. ROTHSTEIN, A., A. D. HAYES, D. H. JENNINGS AND D. C. HOOPER. *J. Gen. Physiol.* 41: 585, 1958.
42. SCHWARZENBACH, G. *Anal. Chem.* 32: 6, 1960.
43. VALLEE, B. L. This symposium.
44. VALLEE, B. L., J. A. RUPLEY, T. L. COOMBS AND H. NEURATH. *J. Biol. Chem.* 235: 64, 1960.
45. WILLIAMS, R. J. P. *Biol. Rev.* 28: 381, 1953.
46. WILLIAMS, R. J. P. *Nature, London* 177: 304, 1956.
47. WILLIAMS, R. J. P. In: *The Enzymes*, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1959, vol. 1, p. 391.
48. WILLIAMS, R. J. P. This symposium.

DISCUSSION

Effect of certain transition elements on cholesterol biosynthesis[1]

GEORGE L. CURRAN AND DANIEL L. AZARNOFF

Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri

THE EFFECTS OF FOUR TRANSITION ELEMENTS on the incorporation of C^{14}-labeled acetate into cholesterol and fatty acids by surviving rat liver are shown in Table 1. Magnanese increases the incorporation of radioactivity into cholesterol markedly and into fatty acids slightly (3). Chromium stimulates incorporation into

[1] Studies conducted by the authors were supported by U.S.-P.H.S. Grants H-1947 and H-4882.

TABLE 1. *Effects of Transition Elements on Incorporation of C^{14}-Labeled Acetate Into Cholesterol and Fatty Acids*

	Cholesterol Biosynthesis	Fatty Acid Biosynthesis
Manganese	++++	+
Vanadium	−−−−	−
Chromium	++++	++++
Iron	−−	−

+ = Increase. − = Decrease.

both cholesterol and fatty acids greatly. Vanadium inhibits the synthesis of cholesterol to a great extent and the formation of fatty acids slightly. Iron has a moderate depressant action on cholesterol synthesis *in vitro* and a lesser effect on fatty acid synthesis.

The action of iron can be demonstrated only *in vitro* and only with iron in its ferrous state. Manganese, vanadium and chromium, on the other hand, show this effect in the intact animal as well as *in vitro*. In addition, the oxidation state of these three metals does not seem to be critical as the noted effects can be demonstrated with chromous, chromic and chromate preparations, with manganous and permanganate compounds, and with vanadium as vanadyl salts and as the vanadate. These findings do not infer that all oxidation states of the metals are active, but merely that in the presence of viable tissue some of the metal can be converted to its active form. The reports of Cotzias (2) and Schwarz and Mertz (8) suggest that the active forms might be Mn^{+3} and Cr^{+3}. No data are available for vanadium.

As can be seen in Table 1 chromium stimulates both cholesterol and fatty acid synthesis while manganese and vanadium act primarily on cholesterol biosynthesis. It would thus seem that the site of action of chromium differs from that for manganese and vandium. One possible explanation exists in the studies of Schwarz and Mertz (8). These workers have identified Cr^{+3} as the Glucose Tolerance Factor, a dietary agent necessary to maintain a normal glucose tolerance in the rat. It thus seems possible that the indiscriminate stimulating effect of chromium on cholesterol and fatty acid synthesis is mediated not by a direct effect on these systems *per se* but, rather, through a stimulating effect of chromium on carbohydrate metabolism with the resultant production of such cofactors for lipid synthesis as reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide.

A further suggestion of the differing mode of action of chromium as compared to vanadium and manganese is found in studies of the antagonistic actions of these metals. In Table 2 we see that, whereas an equimolar concentration of vanadium results in depression of cholesterol synthesis in the presence of chromium, it takes a two mole equivalent of vanadium to just balance the stimulatory effect of manganese.

The demonstrated antithetic action of manganese and vanadium on cholesterol biosynthesis suggests that the intrahepatic concentration of these elements could comprise a natural hepatic mechanism for the homeostatic control of cholesterol synthesis (3). To validate such speculation one must know that both metals are present in the liver microsomes, the site of the enzyme systems for cholesterol synthesis. Although manganese preferentially accumulates in mitochondria, it is present in the microsomes (7). Current investigations in our laboratory show that vanadium accumulates in microsomes as well as other hepatic subcellular particles. Studies to correlate the rate of microsomal cholesterol synthesis with the microsomal manganese-vanadium content ratio are in progress.

Such studies, of course, indicate only that, when vanadium is present in the microsomes, an endogenous mechanism for cholesterol synthesis control exists. The problem remains as to whether in the general animal population, including man, vanadium is a normal constituent of the body. But at least we can say that when vanadium is available in the diet or environment such a mechanism is possible. In support of this thesis, the study by Lewis (6) is suggestive. He found lowered serum cholesterol levels in an occupational group chronically exposed to vanadium dust.

The exact site or sites of the competitive manganese and vanadium effect on cholesterol biosynthesis remains unknown. However, some localization of the vanadium action has been made (1). As Table 3 demonstrates, vanadium has no effect on the conversion of labeled squalene to cholesterol whereas it does depress cholesterol synthesis from either acetate or mevalonic acid. Synthesis proceeds normally through the formation of β-hydroxy-β-methylglutaryl-Coenzyme A and thus the inhibitory effect of vanadium must lie at some reaction between mevalonic acid and squalene. The studies of Tchen (9) suggest that manganese may be the preferred divalent cation cofactor for mevalonic kinase in yeast.

This summary has been confined to the effects of these metals on cholesterol biosynthesis. A review of the more general biological actions of vanadium (4) and a report of its specific effects on sterol balance (5) have recently been published.

TABLE 2. *Antagonistic Actions of Metals on Cholesterol Biosynthesis*

	Cholesterol Biosynthesis
Manganese	++++
Manganese + 1 Vanadium	+++
Manganese + 2 Vanadium	±
Chromium	++++
Chromium + 1 Vanadium	−−−

+ = Increase. − = Decrease.

TABLE 3. *Effect of Vanadium on Incorporation of Labeled Substrates Into Cholesterol*

Substrate	Compound Isolated	Vanadium 10^{-3} M % of Control Radioactivity
Acetate-1-C^{14}	β-Hydroxy-β-methylglutaric acid	165
Acetate-1-C^{14}	Squalene	33
Acetate-1-C^{14}	Cholesterol	23
Mevalonate-2-C^{14}	Cholesterol	14
Biosynthetic C^{14} Squalene	Cholesterol	99

REFERENCES

1. AZARNOFF, D. L. AND G. L. CURRAN. *J. Am. Chem. Soc.* 79: 2968, 1957.
2. COTZIAS, G. C. AND A. J. BERTINCHAMPS. *J. Clin. Invest.* 39: 979, 1960.
3. CURRAN, G. L. *J. Biol. Chem.* 170: 765, 1954.
4. CURRAN, G. L. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 216.
5. CURRAN, G. L., D. L. AZARNOFF AND R. E. BOLINGER. *J. Clin. Invest.* 38: 1251, 1959.
6. LEWIS, C. E., *A. M. A. Arch. Indust. Health* 19: 419, 1959.
7. MAYNARD, L. S. AND G. C. COTZIAS. *J. Biol. Chem.* 214: 489, 1955.
8. SCHWARZ, K. AND W. MERTZ. *Arch. Biochem. Biophys.* 85: 292, 1959.
9. TCHEN, T. T. *J. Biol. Chem.* 233: 1100, 1958.

DISCUSSION

A physiological role of chromium(III) in glucose utilization (glucose tolerance factor)

KLAUS SCHWARZ AND WALTER MERTZ

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

RECENT FINDINGS from our group have shown that trivalent chromium plays a particular role in the normal utilization of glucose (7). The element, in very small amounts, seems to have a physiological function. In the rat it is required as an essential bioelement to maintain normal glucose metabolism; effective levels of chromium complexes are found as normal dietary constituents.

This discovery has come about in the following way: some years ago we were interested in the efficiency of glucose utilization in animals undergoing dietary liver necrosis (8). It was observed that glucose tolerance in rats was strongly dependent on the nature of the diets fed, and that there obviously existed a hitherto unrecognized, specific dietary factor which was necessary for maintenance of normal glucose clearance from the blood (5, 6). This material was contained in natural rations, for instance, in kitchen scraps or in McCollum's wheat casein diet, while many of the artificial semipurified laboratory diets were deficient in it. Animals kept on the latter removed glucose only half as fast as those on optimal diets. Since no other deficiency symptoms were observed at that time, the missing agent was designated "glucose tolerance factor" (GTF),[1] and attempts were made to identify it chemically.

Glucose tolerance is a function which results from a balance of many variables within the organism. The homeostatic mechanisms through which a constant blood glucose level is maintained involve the gut and the liver as "sources" of glucose, the peripheral tissues as the main sites for assimilation of the latter from the blood stream to store it as glycogen or convert it into fat, with practically all tissues of the organism as sites of glucose catabolism. Very efficient and specialized controls and regulatory systems act on these different sites. The best known of the hormonal mechanisms is the island apparatus of the pancreas, producing insulin and glucagon. The adrenals, the pituitary, and other coordinated or controlling centers are involved as well. Incidentally, the delay in glucose clearance from the blood stream is the earliest and most sensitive symptom of diabetes in the human. To measure glucose tolerance, glucose is applied either by mouth or by intravenous injection, and the clearance of the excess sugar from the blood stream is followed through repeated subsequent blood glucose determinations.

In our experiments over the last six years we have used exclusively the intravenous glucose tolerance in order to avoid possible differences in the rate of absorption. Male

[1] Indeed, the impairment of glucose tolerance at first was considered to be a sign of the latent phase of dietary necrotic liver degeneration (8). It was believed that Factor 3, which prevents liver necrosis, was responsible for the glucose tolerance effect since enriched concentrates of Factor 3 contained GTF active material, apparently as a contaminant. In the course of our extensive fractionation project aimed at the identification of Factor 3, we then succeeded in separating the glucose tolerance active principle chemically from the latter. Side fractions, highly effective with respect to glucose tolerance but inactive against necrotic liver disease, were obtained from pork kidney powder in the course of the purification of Factor 3 in pilot plant scale. Thus, we recognized that GTF was an entity of itself (5, 6). Factor 3 was identified in 1957 as an organic *selenium* compound of high biological potency. It has been clearly established that selenium compounds do not affect glucose tolerance (5) and that GTF active chromium complexes do not influence dietary necrotic liver disease.

rats of approximately 150–200 gm of body weight were kept on the various diets. Glucose removal rates from the blood were established after injecting 125 mg of glucose per 100 gm of body weight following an 18-hour fast, and by measuring glucose levels at 15, 25, 35, and 45 minutes thereafter or at other suitable intervals. This is a relatively tricky and laborious technique, but it has been of great value in our studies. If the excess glucose levels in the blood are plotted against time on a semilogarithmic scale, an approximate straight line is obtained (Fig. 1). In other words, over the period of the test the same percentage of the remaining excess glucose is removed for any given interval of time. By a simple calculation one obtains the rate of glucose disappearance. We found that normal, male Sprague-Dawley rats on an optimal diet removed from their blood stream approximately 4 to 4.5 per cent of the excess glucose per minute. On a variety of artificial diets, for instance, on casein sucrose rations, on diets which contain Torula yeast as protein source, etc., the removal rates amounted on the average to 2.8 per cent per minute or less (Table 1, part *A*). We wish to emphasize that several of the pelleted commercial laboratory feeds for rats and other animals lead to impaired glucose removal rates (3). They are deficient in GTF. We have used Purina laboratory chow to great advantage over the last few years as the basal deficiency diet for our animal experiments on GTF, and we are convinced that in many laboratories work is being done on glucose utilization and similar problems with animals which are GTF deficient by coincidence.

For testing of GTF, we have devised a curative assay which has the advantage of being faster than any prophylactic dietary test. Animals were kept on the GTF deficient rations until their glucose tolerance was impaired. Fractions of materials to be tested for activity were given in a single dose by stomach tube to such pretested, deficient rats at night and glucose tolerance was measured anew 18 hours later, i.e., on the following morning (Table 1, part *B*). GTF activity is widely distributed in nature. It is present, for instance, in Brewer's yeast, in kidney powder, in liver, etc.

For fractionation procedures, enzymatic digests of Brewer's yeast and also acid hydrolysates of pork kidney powder were used as main starting materials. The factor was found to be water soluble, stable against treatment by acid and alkali and extractable by a variety of solvents such as phenol, isobutanol and amyl alcohol. It could be absorbed on ion exchangers, and the extractability by solvent systems permitted fractionation by means of counter current distribution. In all these techniques, the active ingredient showed a distinctly cationic property. Fractionation of GTF was seriously handicapped by the fact that crude, as well as highly concentrated fractions underwent inactivation upon storage (Table 1, part *C*). The loss of activity was not due to autoxidation. It could not be prevented by the exclusion of O_2 or by reducing agents such as cysteine or ascorbic acid. At the time, the nature of this inactivation process was a mystery to us. In retrospect, we remembered that in some cases the process of inactivation was accompanied by the appearance of a very faint, brown precipitation which adhered to the walls of the bottles. When trivalent chromium was identified as the active ingredient, these precipitates were shown to give a strong benzidine-blue reaction. They most likely consisted of inert, colloidal chromium oxide.

In sharp contrast to the instability of GTF in concentrates during storage was the fact that the biologically active material in crude and in highly concentrated

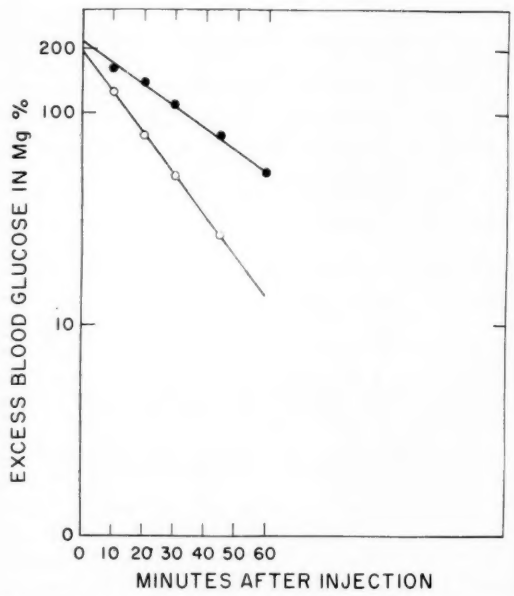


FIG. 1. Effect of diet on glucose tolerance. *Steep curve:* normal diet, removal rate 4.1 ± .4% per minute; *upper curve:* glucose tolerance factor deficient basal, removal rate 2.0 ± .4% per minute.

TABLE 1. *Assay and Properties of Glucose Tolerance Factor*

	Dose, mg/100 gm Body Wt.	No. of Animals	Before Supplement	After Supplement
A. Different diets				
Optimal diet (McCollum's wheat-casein ration)		48		4.3
GTF-deficient basal diet (Purina)		150	2.8	
B. Curative assay				
Controls (water)	1	4	2.3 ± 0.2	2.4 ± 0.2
3/4184, GTF preparation from pork kidney powder	1	6	2.0 ± 0.2	4.1 ± 0.4
C. Spontaneous inactivation				
3/4184, 8 months later	1	5	2.5 ± 0.2	2.7 ± 0.4
D. Stability to ashing				
3/4184, wet ashed	1	4	2.4 ± 0.3	4.4 ± 1.5

preparations was stable against wet ashing with HNO_3-H_2SO_4 mixtures (Table 1, part *D*). When this procedure was followed by a brief fractionation with phenol, biopotency could be recovered practically quantitatively. This clearly indicated the involvement of a trace element. Screening of various elements for glucose tolerance factor activity was then undertaken. A total of 47 different elements were assayed either individually or by testing of mixtures or combinations (7). Most other elements of the periodic system could be eliminated from screening for other reasons. Many do not have cationic properties and others are contained in our diet. The identification of trivalent chromium as the active ingredient was not easy. At first we tested hexavalent chromium and found it to be inactive. For a time it was thought that vanadium was the effective element, but we could not demonstrate vanadium in active, purified fractions. Then it was detected that type and cleaning of glassware had a decisive effect on the activity of certain combinations of elements screened for biopotency. Solutions of vanadyl salts, but also titanyl salts and similar agents were active when made up in glassware mechanically washed after a dichromate-sulfuric acid bath. These effects were not obtained with glass boiled in detergent and washed carefully. It finally became apparent that trivalent chromium was the common denominator of those mixtures and solutions which were effective.

Characteristic results with chromium complexes are presented in Table 2. It is obvious that very stable chromium complexes are inactive, evidently because the element is biologically unavailable. Thus, the acetylacetonate and also most ethylenediamine complexes are inactive. Good results are obtained with the oxalate, the urea, the salicylate, and also with the partially hydrolyzed biguanide complexes. It seems as if it were necessary to supply the element in a complex of intermediate stability. In most cases a dose level of 20 μg of chromium per 100 gm of body weight was sufficient for an optimal response in the curative GTF assay. Comparison of the tris-biguanide with the bis-biguanide hydroxo-aquo complex shows that partial hydrolysis of a complex may enhance its biological availability. There is also evidence that olation does not necessarily preclude biological activity, at least not as long as it involves not more than two chromium atoms.

The determination of chromium in dietary ingredients and also in tissues poses problems which have not yet been overcome entirely. Many of the values in the older literature are unsatisfactory, especially if the results indicate there is "no chromium". Analysis of those ingredients which have been used in our laboratory gave levels of between 1 and 10 ppm chromium. Elsewhere, chromium levels of .002 to 2 ppm are quoted for human food products (1). However, just as different chromium complexes show different biopotencies, it was found that the chromium in various natural dietary ingredients is bound differently. In some it is biologically inert. In particular, nucleic acids and their derivatives seem to bind chromium in such a way that it is without GTF effect following oral application. In this connection it must be mentioned that ribonucleic acid fractions have been obtained from beef liver which contain up to 0.8 mg of the element per gm (10). From unpublished results obtained previously by our group, it appears as if the

TABLE 2. *GTF-Potency of Chromium(III) Complexes*

	Dose, mg/100 gm Body Wt.	No. of Animals	Glucose Removal Rates	
			Before Supplement	After Supplement
Chromium(III) acetylacetonate, $[Cr(C_5H_7O_2)_3]$	.02	4	1.7 ± .5	1.5 ± .4
Bis-ethylenediamine complex, $cis[Cr(en)_2Cl_2]Cl$	.02	4	1.7 ± .4	2.3 ± .4
Chromium(III) perchlorate, $[Cr(H_2O)_6](ClO_4)_3$	.02	2	1.8	3.0
Chrome alum, $K_2(SO_4)\cdot Cr_2(SO_4)_3\cdot 24H_2O$	.05	4	1.5 ± .2	3.3 ± .7
Potassium trioxalato chromate-(III), $K_3[Cr(C_2O_4)_3]\cdot 3H_2O$	.02	4	1.5 ± .2	3.9 ± .8
Hexa-urea chromium(III) chloride, $[Cr(C_2ON_2H_4)_6]Cl_3$	.02	4	1.5 ± .2	4.1 ± 1.0
Chromium(III) salicylate, $[Cr(C_7H_5O_3)_2(H_2O)(OH)]$	.02	16	1.9 ± .1	3.6 ± .4
Tris-biguanide chromium(III) chloride, $[Cr(C_2N_5H_6)_3]Cl_3$	.02	4	1.8 ± .1	2.9 ± .7
Bis-biguanide chromium(III) sulfate, $[Cr(C_2N_5H_6)_2(H_2O)\cdot(OH)]SO_4$	.02	8	2.1 ± .3	4.1 ± .6

TABLE 3. *Effect of Chromium(III) and Insulin on Glucose Uptake by Fat Tissue in Vitro**

Insulin	No. of Rats	Glucose Uptake		Increase of Uptake	
		Without	With Cr(III)		
Milliunits/flask		*μg/100 mg/h*†		*μg/100 mg/h*†	*% of basal uptake*
0	10	16 ± 5	12 ± 3		
.01	10	23 ± 3	31 ± 5	8 ± 5	+35
.1	10	36 ± 4	52 ± 5	16 ± 6	+44
1.0	10	41 ± 6	71 ± 9	30 ± 6	+73

* .1 μg/flask as chromalum.
† Mean and standard error.

TABLE 4. *Dose-Response of Epididymal Fat Tissue to Chromium(III) Supplementation in Vitro**

Supplement	Increase of Uptake over Unsupplemented Controls	
μg Cr(III)/ flask†	μg/100 mg/h‡	% of basal uptake
0		
.0001	−1 ± 8	
.001	+7 ± 6	+28
.01	+28 ± 6	+88
.1	+30 ± 6	+94
1.0	+14 ± 6	+44
10	+14 ± 8	+54

* All flasks contained 1 milliunit insulin.
† Added as neutralized solution of chromalum.
‡ Mean and standard error.

liver plays an important role in chromium(III) storage, activation, and release.

There are some reports indicating that with modern methods the element is found throughout the organism. The chromium contents of the ash of normal human tissues has been analyzed spectroanalytically (2). More recently a thorough survey of the chromium contents of normal tissue has been made by I. Tipton. It is noteworthy that tissues of the newborn are especially rich in chromium; there is a decline till early adulthood (9). In many tissues chromium values have been found which are quite compatible with the dose levels which are needed to cure GTF deficiency in rats. These levels are also similar to those needed in *in vitro* systems in which chromium has been found to be biologically effective (see below).

A major metabolic pathway for excess glucose in the course of a glucose tolerance test is its conversion into fat. In order to define the site of action of GTF, glucose uptake was measured using the epididymal fat tissue technique (4). The epididymal fat body of rats can be used readily for metabolic studies. It can be handled much like other tissues. The technique has been used extensively to assay insulin and insulin-like substances. While incorporation of glucose into this tissue is relatively small in the absence of insulin supplements, it can be stimulated considerably by the addition of physiological levels of the hormone (Table 3). In our experiments, each flask contains ca. 100 mg fat tissue and 2 ml medium. With insulin levels of 10 or more milliunits per flask, the stimulation of glucose uptake is so great that supplementation of chromium does not further enhance it. At lower, physiological insulin levels glucose uptake is greatly increased by .1 μg of chromium(III). The results in Table 3 were obtained with neutralized chromalum. When 1 milliunit of insulin was supplied and the chromium levels were varied (Table 4), an optimum effect of chromium supplementation was obtained with as little as .01 μg of the element per flask. Glucose uptake is almost doubled. Identical results have been obtained with C_{14}-labeled glucose, by means of which it is demonstrable that the glucose is converted into fat. In the absence of insulin supplements chromium does not act on this system. This seems to indicate that the element acts either directly in conjunction with the hormone or at a site which is close to that of insulin. Whether a stoichiometric relation exists between insulin and chromium cannot be concluded from the data on hand. It may be mentioned that small amounts of chromium have been detected in all samples of insulin we have analyzed thus far.

The mode of action of insulin has been a controversial issue over the years. Some believe that it acts by accelerating the entry of glucose into the cell, while others think that it has catalytic effects in enzyme systems. From preliminary results it appears that chromium does not only promote the incorporation of glucose into fat cells, but also the entry of galactose. Since galactose is not metabolized to any degree by fat tissue, these experiments seem to indicate that chromium and insulin jointly affect the entry of sugars into the cell. However, it seems possible that the argument of "entry" mechanisms vs "catalytic" effects is not really at issue because "catalytic" mechanisms undoubtedly also are involved in glucose transport through the cell membrane. Hence, a thorough analysis of the possible effects of chromium on enzyme systems participating in glucose assimilation and metabolism is indicated before a final answer as to the mechanism of action of the element in glucose utilization can be given. It seems possible that some of the existing controversies in the field of glucose metabolism may be explainable by the presence or absence of effective levels of the element hitherto not realized to be physiologically important. One may think of the controversy about the effects of insulin on the hexokinase reaction. The possible role of chromium in the phosphoglucomutase reaction is discussed elsewhere (11).

The finding of the role of chromium in glucose utilization opens up a great number of interesting problems and questions. Not the least of these, of course, is whether defects of chromium supply or metabolism may not be etiologically connected to diabetes, especially those forms of diabetes which are known to have normal or even elevated insulin levels in the blood stream. It appears likely that for the handling of chromium the organism has special mechanisms of absorption, transportation, storage, activation, and possibly also elimination. With reference to the theme of this monograph, these mechanisms must constitute highly specialized and "complex" instances of metal-binding in biological systems.

REFERENCES

1. GRUSHKO, Y. M. *Biokhimiya* 13: 124, 1948.
2. MERTZ, W. AND K. SCHWARZ. *Arch. Biochem. Biophys.* 58: 504, 1955.
3. MERTZ, W. AND K. SCHWARZ. *Am. J. Physiol.* 196: 614, 1959.
4. MERTZ, W. AND K. SCHWARZ. *J. Biol. Chem.* 236: 318, 1961.
5. SCHWARZ, K. A Note on the Glucose Tolerance Factor (GTF), a New Dietary Agent. In: *Liver Function*, edited by R. W. Brauer. Washington, D. C., American Institute of Biological Sciences, 1958, p. 535.
6. SCHWARZ, K. AND W. MERTZ. *Arch. Biochem. Biophys.* 72: 515, 1957.
7. SCHWARZ, K. AND W. MERTZ. *Arch. Biochem. Biophys.* 85: 292, 1959.
8. SCHWARZ, K. AND W. MERTZ. *Metabolism* 8: 79, 1959.
9. TIPTON, I. H., The Distribution of Trace Metals in the Human Body. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 27.
10. WACKER, W. E. C. AND B. L. VALLEE. *Fed. Proc.* 18: 345, 1959.
11. These Proceedings. [Discussion to Dr. Najjar's paper.]

DR. DOUGLAS SURGENOR (University of Buffalo School of Medicine): It would appear, from your evidence, Dr. Cotzias, that the manganese is interacting with transferrin—the iron-binding protein of the plasma. In support of this, J. K. Inman, in work at Harvard with J. L. Oncley (unpublished), was able to prepare the Mn^{+3} complex of transferrin. This would be consistent with your electrophoretic observations. It is also noteworthy that transferrin apparently can discriminate between magnesium and manganese on the one hand but exhibits good affinity for iron and copper. These are, of course, *in vitro* results, and I am aware that there is no evidence that transferrin is involved in the *in vivo* transport of copper.

DR. COTZIAS: We do not feel that we have resolved the problem of protein carriers for iron versus manganese. We find different pKs for manganese in plasma than in transferrin, but we have not isolated two different proteins as yet.

DR. CURRAN: Did vanadyl sulfate change the disappearance curve of the manganese?

DR. COTZIAS: Vanadyl sulfate did not change the disappearance curve of manganese. Only manganese changed manganese. None of the other elements did that we tried and the internal distribution of manganese remained constant when vanadyl salts were administered.

DR. THOMAS JUKES (American Cyanamid Company, New York, N. Y.): The lack of any resemblance between manganese and magnesium in the deficiency syndrome in animals has been evident for a long time. Manganese deficiency and magnesium deficiency were both studied by McCollum in rats in the 1930's on diets in which the other metal respectively was supplied. The same holds true for chickens. These animals are subject to acute deficiencies of either manganese or magnesium on diets which contain the other metal in ample amounts. There is no resemblance at all between the two deficiencies in appearance or characteristics or interchangeability, so I think the burden of proof is in the opposite direction.

DR. SNELL: I would like to add to Dr. Jukes' remarks to point out that since manganese deficiency symptoms are determined in animals amply provided with magnesium and, conversely, you automatically exclude as limiting systems all of those systems which can function equally well with either one of the two elements. For this reason, you shouldn't expect the syndromes to have any resemblance to one another or the metal ions to show any mutual replaceability when tested under these conditions. This does not exclude the possibility, which I consider a probability, that there are a large number of enzymes in mammals which function interchangeably with Mn^{++} or Mg^{++} as activator.

DR. RUBIN: For many metals, especially iron, the distribution and excretion is very much a function of the carrier.

DR. COTZIAS: We are aware of that and will shortly present experiments (Hughes, E. R. and G. C. Cotzias) showing that globulin-bound radiomanganese pursues the same route as unbound tracer following parenteral injection. The disappearance curves from the blood tend to be slower in the former than in the latter case.

DR. BORG: I have been asked about our work on the manganese porphyrin. I don't think we can say definitely that we have confirmed the identification of a manganese porphyrin. However, the data in hand provide an overwhelming presumption for such a conclusion. We have found tracer radiomanganese injected into man and animals to be incorporated into erythrocytes in a reproducible and characteristic way (Borg, D. C. and G. C. Cotzias. *Nature, London* 182: 1677, 1958). The character of the radioactive component is compatible with its being a protoporphyrin. That is, there is comparable behavior in *in vitro* systems between the compound whose radiomanganese is incorporated *in vivo* and a manganese protoporphyrin containing radioactive tracer that we have synthesized. However, we don't yet have an isolation of the manganese protoporphyrin directly from the red cell. I can say that the manganese that is synthesized by the living animal into the red cell is incorporated in a very different fashion from what one sees when manganese is added *in vitro* either to red cells or to hemolysates. In the latter two cases tracer added *in vitro* can be eluted. Dr. Weed has stated that when he added radiomanganese to a hemolysate, 50 per cent of it was ultrafilterable, and the remainder was not. With dialysis experiments of a similar kind we found—to be sure—that manganese was well bound to hemolysate but that we could elute tracer manganese with carrier manganese or with chelating agents until finally all radioactivity was removed. The manganese that is put in by the body is completely refractory to this same challenge (Borg, D. C. and G. C. Cotzias. *Nature, London* 182: 1677, 1958).

DR. COTZIAS: Isolation of the heme component makes for recovery of the tag with the heme.

DR. PETERS: I think it would be appropriate to call attention to the presence of coproporphyrin III and protoporphyrin in the white matter of the central nervous system as shown by Heinrich Klüver, Cecil Watson and others. Figy and Solomon have demonstrated the presence of these porphyrins in the peripheral nerves. Since manganese becomes incorporated in these porphyrin substances, this might be one of the reasons why the manganese might ultimately find its way into the basal ganglia to cause symptoms of Parkinsonism.

DR. COTZIAS: I am very grateful for this comment because this is, as you know, an operating hypothesis of ours.

DR. SCHWARZ: To Dr. Scheinberg's remarks I would like to comment that one must differentiate very clearly between the amount of a trace element in the diet and that amount of the element which is really biologically available to the animal. This is an important difference which is often overlooked. Just as in living systems, trace elements in nutrition are not free, but bound. In our work we have experienced this with selenium (Schwarz, K. and C. M. Foltz. *J. Biol. Chem.* 233: 245, 1958), and more recently also with chromium (Schwarz, K. and W. Mertz. *Arch. Biochem. Biophys.* 85: 292, 1959). In the latter case, only a small proportion of the element in nutrients is biologically utilized. It is a difficult, but essential and urgent, research proposition to identify the sites and modes of binding for the various essential elements in dietary materials, and to determine which forms are biologically used, and which not. Nucleic acid may be involved besides proteins and carbohydrates.

That trace elements, especially heavy metals, are not absorbed *ad infinitum* by the organism seems to be well established in some cases, for example, for iron. It appears as if each element must be treated separately in that respect, since the organism may use specialized mechanisms for each one of these essential, but potentially dangerous, dietary constituents. Phylogenetically, such discretionary barriers may have developed very early, otherwise life would have been destroyed long before our time.

DR. COTZIAS: When I was stressing that the mode of absorption and the chemical form or valence state are unknown I had exactly this idea in mind.

DR. SCHEINBERG: I think what Dr. Schwarz said is obviously

true but I also think that the figures are such that you could have a large fraction of the metal unavailable, and still deal with a relative excess nutritionally. For a 70 kg man, consisting, let us say of 35 kg of protein, 70 grams of dietary protein per day are amply sufficient. This intake represents something like 0.2 per cent of the body weight of protein. Now this ratio of nutritional supply to body content is such that you could divide it, for these metals, by 10, or 100, or even 1,000, to allow for what Dr. Schwarz referred to as barriers to absorption, or unavailability, and still be far away from deficiency—in fact you would still have an excess. So I think it is possible that toxicity of some metals is not just avoided by chance, but by an active process.

DR. JUKES: I would like to remind you of the extensive literature on the extent to which elements such as manganese and zinc are absorbed by animals, only a very small fraction is absorbed. And also, certain constituents of the diet in some cases prevent the elements from being absorbed at all. Calcium carbonate and calcium phosphate markedly reduce the utilization of manganese and zinc by animals.

DR. COTZIAS: This problem was discussed during a recent symposium (Cotzias, G. C. *Fed. Proc.* 19: 655, 1960).

DR. PETERS: I want to compliment Dr. Borg and Dr. Cotzias on a very excellent piece of work. I think it throws considerable light on a phenomenon which has been observed clinically since the introduction of the phenothiazine compounds, namely that if you give enough of these phenothiazine drugs (chlorpromazine being the first) you will almost invariably induce symptoms of Parkinsonism. Other of the newer phenothiazine derivatives seem capable of provoking extremely serious and often bizarre neurological symptomatology which continues, in some instances, a long time after withdrawal of the particular drug. Now Dr. Borg and Dr. Cotzias have demonstrated that manganese incorporates itself with at least one of these phenothiazine compounds. This raises the possibility that because of the small size of this compound it may cross the so-called "blood brain barrier", perhaps lending itself to a situation where the manganese might be deposited in the basal ganglia to give rise to extrapyramidal symptoms. This is a field of investigation which must be pursued with great vigor because at this time these compounds are being used to a greater extent than perhaps any other drugs when you realize the huge amounts being used in state mental hospitals and by the entire medical profession.

DR. COTZIAS: Dr. Peters, on two occasions you seemed to exhibit insight identical to our own. I don't know whether that puts you into good or bad company. My feeling is that this conference and others like it should promote collaborations and you are proposing the same. The actual reason for Dr. Borg and myself entering this type of work is, indeed, the parallelism between the manganese picture and the phenothiazine picture which you have presented.

DR. ALBERT: Dr. Borg mentioned the oxidation potential of phenothiazines. What is the oxidation-reduction potential of your chlorpromazine? Is it the oxidized or reduced form which combines with manganese?

DR. BORG: Chlorpromazine is added in the reduced form in the reactions with manganese and ferric iron that I mentioned. However, as I indicated earlier, it is likely that the spectrum I presented actually derives from the more oxidized free radical form of chlorpromazine following the transfer of one electron to the metal cation. We have not yet measured the electrode potential of this oxidation, but Karreman and others (Karreman, G., A. Isenberg and A. Szent-Györgi. *Science* 130: 1191, 1959) calculated by LCAO molecular orbital methods that the "K" of the highest filled molecular electronic orbital of reduced chlorpromazine is *minus* 0.217, suggesting an antibonding orbital with a strong monovalent electron donor propensity. Some years ago Michaelis and his colleagues (Michaelis, L., S. Granick and M. P. Schubert. *J. Am. Chem. Soc.* 63: 351, 1941) actually determined by potentiometric titration a normal potential of *plus* 0.83 to 0.88 volts for the oxidation to its semiquinone free radical form on N-methylphenothiazine, which is similar to chlorpromazine except for the absence of ring halogenation.

I am no physical chemist, and at times it is difficult for me to find a sharp differentiation between a charge-transfer complex and any other redox donation of an electron to an acceptor. Maybe Dr. Calvin can clarify this point.

DR. CALVIN: Well, the radical that you described is undoubtedly there. This is due to an actual oxidation of the phenothiazine by the manganese and oxygen together. What the detailed mechanism is—is a question. Whether this oxygen oxidizes the phenothiazine—the manganese stabilizes—or whether the manganese is oxidized and this in turn carries the oxygen to or removes the electron from the phenothiazine—is another question. I don't want to argue the point as to the presence of, or absence of, a charge-transfer complex. There may very well be one there but the free radical signal is not the charge-transfer complex. These are two different things. The free radical signal is almost certainly due to this radical (and also most of your absorption spectrum). Now there may be a little of the charge-transfer complex, as well, in the mixture. I think the chances are there are several things there. There is certainly the free radical which is a free entity. It may be bound by manganese or may not, as the case may be. But there may also be a charge-transfer complex which is not magnetic, in general. The sort of thing that you have seen is undoubtedly this. One could go into a long discussion as to the nature of charge-transfer complexes when they are magnetic and when they aren't. In this case I think it would not be magnetic. The free radical signal that you have is really the phenothiazine radical, not the charge-transfer complex.

DR. BORG: I'm convinced that you are right in concluding that the electron paramagnetic resonance spectrum I showed is mostly that of the free radical component. The idea that we had was that the donation of the electron to the metal in charge-transfer process was sufficiently complete so that the phenothiazine spent a significant fraction of the time in a free radical form.* Apparently this seems unlikely to you.

DR. CALVIN: You shouldn't talk about the free radical and the non-free radical as being in resonance with each other because in the free radical the electrons are unpaired and in the non-free radical the electrons are all paired. These are two states of two different multiplicities and they are not in resonance with each other. They are two different things—two distinct species. I think you probably have them both there. The interesting thing is the curious interaction with manganese and oxygen and phenothiazines. We have seen this same radical made by oxidation with quinone.

DR. BORG: As I say, there is other evidence, some of which I gave before, that we are certainly dealing with a free radical. Indeed, it must be the free radical that gives rise to the color of the reaction systems of which I spoke. Let me remind you that we saw *identical* absorption spectra with both manganese and iron. Yet, were the total color change due to a charge-

* (In a book published after this discussion, Szent-Györgi develops essentially this same argument. Szent-Györgi, A. *Introduction to a Submolecular Biology*. New York: Acad. Press, 1960, p. 69)

transfer spectrum, this would be inexplicable. Furthermore, I did mention the study of a free radical very much like the one we are discussing by Michaelis, Granick and Schubert about twenty years ago. They worked with slightly different phenothiazine derivatives, but their free radical spectra look essentially the same as the absorption spectra of colored compounds that I showed. With all these considerations in mind, would it still be fair to say this, Dr. Calvin: If the compound that gave our spin resonance signal is not a charge-transfer complex—that is to say, there is no stabilization by charge-transfer resonance between a dative and a no-bond form—then at least the reaction that produced it was a charge-transfer reaction? [Note: See the discussion of the "Spontaneous type of charge-transfer" in the recent book of Szent-Györgi (Szent-Györgi, A. *Introduction to a Submolecular Biology*. New York: Acad. Press, 1960, p. 59).]

DR. HASTINGS: Dr. Calvin, would you tell us now what a charge-transfer complex is?

DR. CALVIN: It falls in my lap to do this when I don't really know what it is. I'll try and tell you what I think it is. In general, the charge-transfer complex idea arose from studies of the absorption spectrum of iodine in benzene solutions. This was the work by Hildebrandt and Bennet about 15 years ago. They found that the color of iodine in benzene solutions was unusual. It was a nice brown color and most people didn't look at the near ultraviolet absorption, but they did. They found a rather large bump just on the edge of this benzene absorption. This finally led to the notion of the transfer of charge resulting from the light absorption and the discovery much later of many much more profound interactions between molecules which themselves tend to be oxidizing and reducing agents. A typical one which forms very good charge-transfer complexes, chloranil, is a common one that is used now and is very much under investigation. This is a good oxidizing agent and there is room for two more electrons on it. The reduced form, of course, is the hydroquinone. If you put this together with suitable electron donors such as phenothiazine, for example, if you mix a solution of chloranil and a solution of phenothiazine, you get a precipitate. This precipitate (you can also see it in solution) will be made up of two components, a charge-transfer complex. Now, you can see both components. You can see the component in which the electrons which are in the π-orbitals of one molecule (donor) are also involved in the π-orbitals of the other one (acceptor). The two together form a complex with electrons moving in orbitals common to both, but there are no unpaired electrons in this. The π-orbitals of the two molecules overlap, forming, if you like, a molecular orbital involving both, and the electrons move in both. There are various ways of expressing this but that is the simplest way.

The other type of reaction that happens is a complete and total transfer of one of these electrons from donor to acceptor, making two semiquinones—a pair of semiquinone ion radicals. This is a different thing. You have one extra charge here—a free radical on one quinone and on the other one a positive ion radical in which one electron is missing, i.e., has been transferred to the first quinone. These two are separate and dissociable ions if the solvent has a high enough dielectric constant. They are just like any other ion pair, for example, sodium chloride, except that these are stable free radicals and they separate. Charge-transfer complexes and radical ion pairs are two different things. One is a singlet, ground state, and the other is two doublets. The latter shows two separate electron spins. The former shows no unpaired electron spin. The color of this, the so-called charge-transfer spectrum, is due to the transition in which electrons are transferred from an orbital which is more on one molecule to an orbital which is more on the other one. The excited state may be spoken of as a pair of radical ions in which the two spins are antiparallel. They must be antiparallel—one cannot get a transition from one spin state to another by light absorption. It is prohibited. So that the so-called charge-transfer band, perhaps this one which Dr. Borg spoke of, involves a change from a state of motion in which the electrons are more on this orbital than on this one to one in which one electron is over on the other side and one is on this side but both in antiparallel spin—both in the same orbital—if you like. Therefore, the excited state is a diamagnetic singlet state but the others are a pair of two distinct free radicals. This is about all we can do in the time we have available.

DR. BORG: I want to be as flexible as I can be in incorporating your remarks into some further comments about the phenothiazines. Some of my earlier interpretations may still hold true, but they can be seen in a different light after your very helpful criticism. Thus, there may still be a complex between the metal and the phenothiazine; but if this cannot be a charge-transfer-stabilized complex, then it must be a metal complex with the free radical itself. If the metal is removed *after* this complex is formed, the spin signal and the color both disappear. For example, if the metal is sequestered by a chelating agent this will happen. (Note: In a later private discussion, R. J. P. Williams raised the question of whether certain chelating agents might not quench the free radical by reducing it as a consequence of their stabilizing preferentially the more oxidized forms of the metal cations involved. Hence, in such cases the chelating agent effect *per se* might not be evidence of a metal-free radical complex.) Thus the metal cation may be required for the stabilization of these phenothiazine free radical forms in analogy to the way that Michaelis *et al.* treated the role of the proton in stabilizing their very similar free radicals. In summary, my earlier query regarding the possibility of charge-transfer complexes giving rise to specificity in metal-binding should be supplemented: Is there any reason to expect specificity in the complexing of metals with free radicals?

DR. GRYDER: I wonder whether or not the specificity may be artificial in the sense that the other ions are not oxidizing ions. Have you done any work in which you electrolyzed at the same time or added another oxidizing agent to stabilize the free radical in the presence of another ion?

DR. BORG: No, we have not done that directly; although we have found that peroxide has too high an oxidation potential and forms sulfoxides from phenothiazines rather than free radicals—even in the presence of a metal ion. It is true that most of the cations we tested were in their lowest stable ionic valence states, but several were oxidizing ions. There was cupric ion for one; although it is relatively weakly oxidizing. In addition, we used ions that are capable of oxidation to higher valences. In the same way that manganous ions were reactive in our system under conditions favoring autoxidation, cobaltous and ferrous ions were inert: even under identical conditions of aerobic titration and back-titration. However, manganese also was active if converted to the manganic state *before* addition of the phenothiazine; but I haven't yet used cobaltic ion directly.

DR. COTZIAS: Don, don't you want to say something about the colorless free radicals of these drugs?

DR. BORG: I should like to make some final remarks about another kind of free radical observed with phenothiazines. It has been reported that a very stable free radical is produced by ultraviolet irradiation of chlorpromazine in the absence of metal (Forrest, I. S., F. M. Forrest and M. Berger. *Biochim. et biophys. acta* 29: 441, 1958). However, this radical is colorless

and also lacks a characteristic ultraviolet absorption; so it must not be the free radical we have discussed here. This is pointed up by the fact that when we first irradiate phenothiazines with ultraviolet light and then expose them to the metal ion, we get very different kinds of colored compounds than with unirradiated material. In the cases of both manganese and iron the colored product from irradiated chlorpromazine is much less stable and has a distinctly altered absorption spectrum. Therefore, there may be more than one free radical form of the phenothiazines.

Dr. Curran mentioned that animals given chromium or manganese develop a hypercholesterolemic response but that the elements may be administered in any of several valence states with essentially equivalent results. This is strikingly reminiscent of Dr. Cotzias' experience regarding the kinetics of manganese turnover in the whole animal. With experiments of the type he discussed here, he found that radio-manganese excretion kinetics could be altered profoundly when challenged by loading with carrier manganese. These data also answer Dr. Rubin's earlier query regarding the validity of findings derived primarily from the use of manganous chloride. The challenging manganese can be given as the divalent cation with any one of a number of anions or in valence states ranging from zero (powdered metal implants) to plus seven: all with comparable results (Cotzias, G. C. and J. J. Greenough. *J. Clin. Invest.* 37: 1298, 1958). Apparently, the redox systems of the body are capable of channeling the element into the same common pathway of physiological transport—possibly in the trivalent form suggested by Dr. Cotzias—regardless of the valency of administered material. Dr. Curran's work implies that a similar situation exists with chromium. Quite possibly, one can extrapolate a general ability of living tissue to funnel different valence forms of other essential metal elements, as well, into their respective physiological cycles.

Dr. Gould: If one accepts the attractive hypothesis that the vanadium acts in the intact animal by antagonizing manganese, preventing the conversion of mevalonic acid to squalene, would it not follow that it would antagonize manganese in all its other manifestations and thus produce a state of manganese deficiency. Have you been able to produce anything resembling manganese deficiency by increasing the vanadium to toxic levels?

Dr. Curran: No, we have not, in animals or in humans. We have studied patients with brain tumors of one type or another and some of these patients have received large quantities of oral vanadium for as long as eight months without clinical, chemical, or histological evidence of toxicity. I should have pointed out that the action of vanadium on cholesterol biosynthesis seems to be quite specific. For example, with acetate as substrate for liver slices, the presence of 0.1 millimolar vanadium causes no decrease in the oxygen consumption of the slices. The results when we used dietary 8-hydroxyquinoline to chelate manganese in intact rats were totally different. Here, after a few days, cholesterol biosynthesis was reduced but would return to normal with the addition of manganese. However, if the administration of 8-hydroxyquinoline was continued for ten days, the animals literally fell to pieces. This toxicity, we feel, resulted from involvement of numerous enzyme systems in which manganese was necessary.

Dr. Hastings: Dr. Curran, did anything change with respect to fatty acid synthesis when the vanadium was put into the system?

Dr. Curran: As I showed in Table 1, there is a slight depression in the incorporation of acetate into fatty acids. The depression is not marked, nor is the stimulation by manganese of fatty acid synthesis very marked.

Dr. Hastings: I meant, when the vanadium antagonized the manganese and inhibited the cholesterol synthesis, did the fatty acid increase?

Dr. Curran: In our balance studies in man we found that, while inhibition of cholesterol biosynthesis with vanadium lowered the serum cholesterol, there was a small but statistically significant rise in the serum triglycerides.

Dr. Chenoweth: Dr. Schwarz, do diabetic animals show any improvement in their glucose tolerance curves with chromium administration?

Dr. Schwarz: Chromium has a pronounced effect on diabetic animals, specifically alloxan diabetic rats. We can bring alloxan diabetic rats back almost to normal and we have found that there is a very pronounced effect on ketone body formation and acetonuria.

Calcium metabolism[1]

ROBERT D. RAY, JACK STEVENS, IRVING LYON AND ESTHER D. MENSEN

Department of Orthopedic Surgery, Presbyterian-St. Luke's Hospital, and University of Illinois, Research and Educational Hospitals, Chicago, Illinois

ALTHOUGH THE GENERAL METABOLIC PATHWAYS for calcium have long been known, many of the details concerning mechanisms and kinetics have yet to be worked out. In our laboratory we have been particularly interested during the past 10 years in the uptake of ions such as calcium and strontium, and of carbon by bone and their subsequent release to the body fluids. The approach we have followed has been a "gross quantitative one" involving *in vivo* studies in animals and humans. The principles behind these studies are not new. They were outlined in the early experiments of Schoenheimer and his colleagues (17) and were the subject of a recent paper by Cornfield, Steinfield and Greenhouse (7).

It has been generally accepted since the experiments of Hevesy (13) that there are at least two basic mechanisms by which calcium and similar ions may be taken up by bone from the surrounding body fluids; exchange or "interchange" of ions between the bone salt and the body fluids and accretion or the accumulation of bone salt that accompanies or follows the formation of organic matrix (mineralization of the skeleton). In attempting to correlate these processes with the transport of ions to and from bone, several problems arise. The first concerns the concentration of the various ions in the extravascular spaces of the body. The second is the length of time for exchange between the body fluids and the bone salt to reach a "steady state," that is the "reversibility" of the exchange processes (as opposed to the biological cycle of accretion and resorption). A third problem is the distinction, if any, between the uptake of ions by living as opposed to dead bone, in other words, the role played by metabolic processes in the formation and remodeling of the skeleton.

Concerning the first problem, it has been assumed in the past that the concentration of calcium ions in the extravascular fluids is roughly 70 per cent that of the plasma. However, Engel, Joseph, and Catchpole (9) have shown by liquid junction dilution potentials that the Gibbs-Donnan equilibrium and the law of mass action may be used to determine the electrolyte distribution between blood and connective tissues. Their studies indicated that the concentration of calcium ions in cartilage may be as high as three times that of blood. Using the Donnan ratio for sodium ions to calculate the corresponding ratios for divalent ions such as calcium and strontium, Lyon in our laboratory has suggested that the ratio for the extravascular-extracellular fluids is 1.90 compared with blood and for the water content of bone 2.28 to 2.37 or a mean figure of 2.30 instead of the previously assumed value of 1.43. If these calculations prove to be correct, the figure for the relative volume of the exchangeable space for bone-seeking ions such as calcium and strontium will have to be revised.

The second problem, the length of time for exchange between the body fluids and the bone salt to reach a relatively steady state, has been studied *in vitro* by Neuman and his associates (4, 11, 15), in dogs by Marshall, Rowland and Jowsey (14) using quantitative radioautographs, and in phosphorus deficient rats by Copp *et al.* (6). All of these studies suggest that the original assumption of Bauer, Carlsson and Lindquist (1, 2), that the exchangeable fraction of the bone salt is in rapid equilibrium with the body fluids, represents only a first order approximation. Recent clinical studies with Sr^{85} in our laboratory tend to support this conclusion in some cases at least.

By way of review for those unacquainted with the studies of Bauer and co-workers, following intravenous administration of a bone-seeking isotope, it is assumed that the portion remaining in the body may be divided between two major compartments: that in the exchangeable compartment, the diffusible fraction, and that removed from this compartment during the course of skeletal mineralization, the accreted isotope. Furthermore, if one assumes that the volume of the "diffusible" compartment and the rate of accretion are constant and also that there is an interval between uniform distribution of the isotope throughout the exchangeable compartment and mobilization or recycling of accreted isotope by skeletal remodeling, it should be possible from the curve for plasma concentration of the isotope and the curve for cumulative excretion to determine the two unknown constants, namely the volume of the exchangeable compartment and the rate of skeletal accretion. However, if one uses the equations developed by Bauer and associates to calculate the values for accretion and the relative volumes of the exchangeable compartment at successive intervals after administration of a bone-seeking isotope such as Sr^{85}, it can be shown that in many patients a relatively steady state is not established until 3½ to 4 days following administration of the isotope and, in some patients, it is not established (approximated) in the experimental period (Figs. 1 and 2).

[1] The studies reported in this paper were supported by United States Atomic Energy Commission contract At-(11-1)-507.

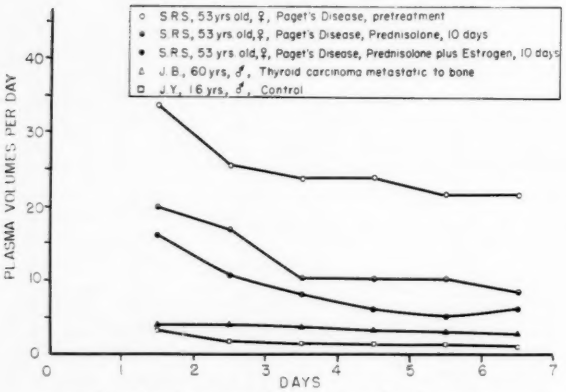
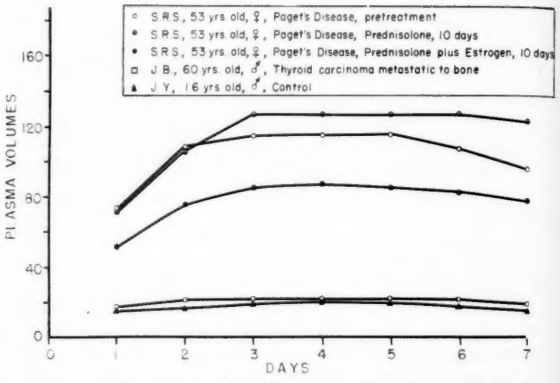

S.R.S., 53 yrs old, ♀, Paget's Disease, pretreatment
S.R.S., 53 yrs old, ♀, Paget's Disease, Prednisolone, 10 days
S.R.S., 53 yrs old, ♀, Paget's Disease, Prednisolone plus Estrogen, 10 days
J.B., 60 yrs, ♂, Thyroid carcinoma metastatic to bone
J.Y., 16 yrs, ♂, Control
PLASMA VOLUMES PER DAY
DAYS

FIG. 1. Calculated *a* values from Sr^{85} studies in patients; *a* is the accretion rate for Sr^{85} expressed in plasma volumes cleared per 24 hours.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S.R.S., 53 yrs old, ♀, Paget's Disease, Prednisolone plus Estrogen, 10 days
J.B., 60 yrs. old, ♂, Thyroid carcinoma metastatic to bone
J.Y., 16 yrs old, ♂, Control
PLASMA VOLUMES
DAYS

FIG. 2. Calculated E values from Sr^{85} studies in patients; E is the relative size of the exchangeable "compartment" or the relative volume of distribution of "diffusible" Sr^{85} expressed as plasma volumes.

Another aspect of the same problem is the "reversibility" of the exchange process. To study this problem, one tibia was removed from each of a group of young growing rats, killed by repeated freezing and thawing, and then implanted under the sheath of the rectus abdominis. One week later the animals were injected with trace amounts of carrier-free Ca^{45}, and at intervals thereafter sacrificed in groups of 6 and the isotope content of the plasma and of the dead and living bone was determined.

Before discussing the results of these studies, a few words concerning the dead bone should be in order. There was a gradual *decrease* in the wet weight of the dead tibia between the time of the original operation and autopsy (Fig. 3) (as opposed to an *increase* in wet weight of the living bone). There was, however, no change in the percentage composition of the dead bone (Fig. 4). [Living rat bone shows a gradual decrease in percentage water and an increase in percentage ash content with increasing age, the percentage organic composition remaining relatively constant (Fig. 5).] On histological sections there was no evidence of nuclear material in the bone lacunae although there was degenerated nuclear material in the epiphyseal and articular cartilage. There were no remaining osteoblasts nor was there any evidence of new bone formation.

The results of the Ca^{45} studies indicated that the initial peaks in uptake for both living and dead cortical, and living and dead cancellous bone were reached within the first four hours. It has been assumed that the initial peak represents establishment of a steady state between the exchangeable fraction of the bone salt and the body fluids. If this were true and if the isotope uptake of dead bone were accounted for solely by exchange then the subsequent Ca^{45} concentration of the dead bone should fall with the decreasing isotope concentration of the plasma, but this was not the case. There was a gradual *increase* in isotope content of the dead cortical bone over the ensuing 60 days, in spite of decreasing plasma activity, and although there was a slight decrease in Ca^{45} activity in the dead metaphyseal bone, the curve did not parallel the plasma activity curve (Fig. 6).

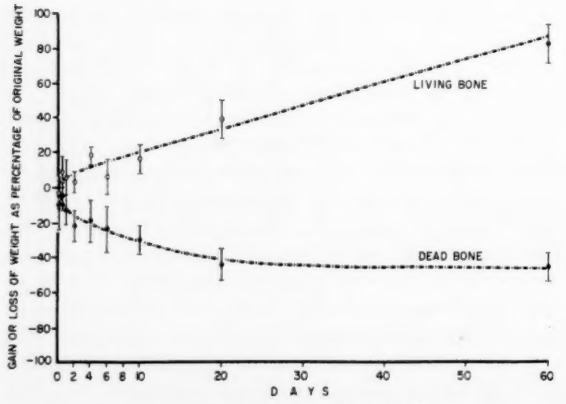


FIG. 3. Change in wet weight of tibia.

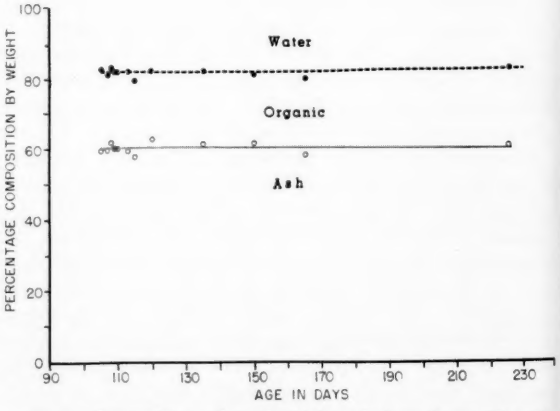


FIG. 4. Composition of dead tibial cortical rat bone. The distance between the bottom of the figure and the stippled line represents the percentage of ash; between the stippled line and the dashed line, the percentage of organic matter; between the dashed line and the top of the figure, the percentage of water.

Can the uptake of isotope by dead bone be explained on the basis of surface accumulation of inorganic salt without coincident new matrix formation? This should result either in an increase in total weight of the bone or a change in composition. The results of an earlier experiment suggested that there was a gradual increase in the percentage ash composition of dead bone in contact with the body fluids (16). This was not verified in the present experiment (Fig. 5) although an increase within the limits of accuracy of weighing and biological variation is possible. The only other explanation that remains is that although initial uptake of Ca^{45} by exchange may be rapid, the establishment of an equilibrium between the body fluids and the radioactive ions in the exchangeable fraction of the dead bone salt may take a long time.

If dead bone can pick up Ca^{45} from the body fluids as rapidly as living bone, can one distinguish between the two processes by isotope studies? The first difference is due to the relative amounts of isotope accumulated (although this may be simply a function of the relative surface areas exposed to the body fluids). In the experiment just cited, the ratio of Ca^{45} activity of dead to living *cortical* bone was approximately 15 per cent and for dead to living *cancellous* bone around 30 per cent (Fig. 7) within the first few days. The second major difference is that bone undergoing rapid remodeling, such as living metaphyseal bone, has a peak in isotope uptake followed by early mobilization and recirculation of the isotope to the body fluids. (This was shown in the current experiment and also previously by Bauer and co-workers.) Apparently, appreciable amounts of the isotope are mobilized from dead bone only with revascularization and resorption of the graft, a slow process compared with the normal remodeling of cancellous bone. In a way dead bone resembles cortical bone in this regard.

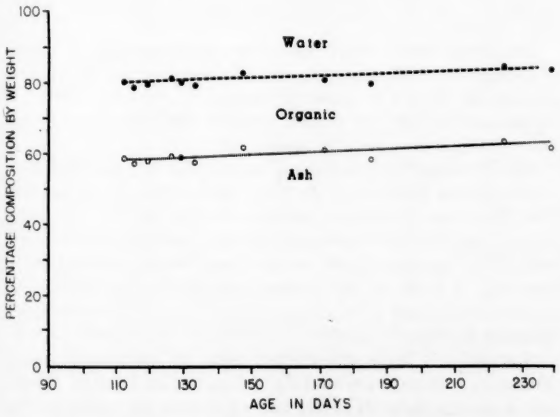


FIG. 5. Composition of living tibial cortical rat bone. The distance between the bottom of the figure and the stippled line represents the percentage of ash; between the stippled line and the dashed line, the percentage of organic matter; between the dashed line and the top of the figure, the percentage of water.

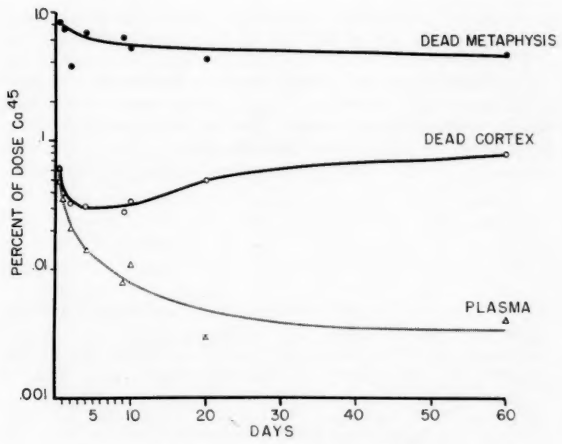


FIG. 6. Ca^{45} content of plasma and dead tibia.

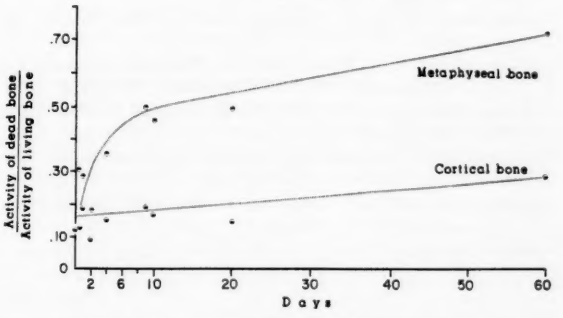


FIG. 7. Ca^{45} content $\frac{\text{dead}}{\text{living}}$ bone.

One final point: Although there may be theoretical limitations to the interpretation of isotope studies of bone metabolism, such clinical and experimental studies as are being carried out currently by Bauer and others (3, 5, 8, 10, 18), cannot help but further our understanding of some of the basic factors involved in bone metabolism. I would like to illustrate this point by citing a single clinical example.

CASE HISTORY

M. S., 63 year old white female, July, 1958.

Chief complaint: Progressively worsening thoracic kyphosis for the past four years and back pain.

History: The patient underwent a surgical menopause at the age of 28. Following this there was a gradual progressive development of generalized osteoporosis, first noticed clinically at the age of 55. Vertebral compression fractures associated with severe back pain started to occur at 59 years of age.

Physical examination: There was a marked thoracic kyphosis and scoliosis with increased A-P diameter of the chest. Neurological examination was normal. The patient's weight was 93 pounds, her height 4 feet 6 inches.

Laboratory data: Urinalysis, hemoglobin and white count were normal. Vital capacity was 50 per cent of normal, and the maximum breathing capacity, 32 per cent of normal. Alkaline phosphatase was 3.9 units, BUN 18 milligrams per cent, serum calcium 9.9 ± 1.2, and serum phosphorus 3.9 ± 0.5 milligrams per cent.

Hospital course: The patient was hospitalized on the metabolic ward through the courtesy of Dr. Theodore Schwartz, and placed

on a standard diet. She was ambulatory. At the end of two weeks, Sr^{85} studies were carried out. During this period the patient was in a slightly negative calcium balance. The patient was then placed on estradiol benzoate for a period of 20 days and again Sr^{85} studies were carried out. During this period the patient went into a slightly positive calcium balance. The results of the Sr^{85} studies are summarized in the accompanying table.

Treatment	Exchange Compartment Plasma Volumes	Accretion Rate Plasma Volumes/24 hrs.
None	8.67	1.20
Estrogen	10.26	0.76

The most feasible explanation for these results is that the estradiol benzoate therapy resulted in a decrease in the rate of bone resorption rather than an increase in the rate of bone formation. This, of course, is consistent with the clinical observation that patients receiving estrogen therapy fail to show rapid formation of new bone even though the progress of the osteoporosis may be arrested.

REFERENCES

1. Bauer, G. C. H., A. Carlsson and B. Lindquist. *Kgl. Fysiograf. Sällskapi Lund, Forh.* 25: 1, 1955.
2. Bauer, G. C. H., A. Carlsson and B. Lindquist. In: *Mineral Metabolism: An Advanced Treatise*, New York: Acad. Press, in press, ch. 14.
3. Bauer, G. C. H. and B. Wendeberg. *J. Bone & Joint Surg.* 41-B: 558, 1959.
4. Boyd, J., W. F. Newman and H. C. Hodge. *Arch. Biochem. Biophys.* 80: 105, 1959.
5. Bronner, F. and R. S. Harris. *Ann. New York Acad. Sc.* 64: 314, 1956.
6. Copp, D. H., J. G. Hamilton, D. C. Jones, D. M. Thompson and C. Cramer. In: *Metabolic Interrelations: Transactions of the Third Conference*, edited by E. C. Reifenstein, Jr. New York: Macy, 1951, p. 226.
7. Cornfield, J., J. Steinfeld and S. W. Greenhouse. *Biometrics* 16: 212, 1960.
8. Dow, E. C. and J. B. Stanbury. *J. Clin. Invest.* 39: 885, 1960.
9. Engel, M. B., N. R. Joseph and H. R. Catchpole. *A.M.A. Arch. Path.* 58: 26, 1954.
10. Fraser, R., M. Harrison and K. Ibbertson. *Quart. J. Med.* 29: 85, 1960.
11. Harrison, G. E., E. Lumsden, W. H. A. Raymond and A. Sutton. *Arch. Biochem. Biophys.* 80: 97, 1959.
12. Heaney, R. D. and G. D. Weadon. *J. Clin. Endocrinol.* 18: 1246, 1958.
13. Hevesy, G. *Intern. J. Appl. Radiation and Isotopes* 2: 85, 1957.
14. Marshall, J. H., R. E. Rowland and J. Jowsey. *Radiation Research* 10: 213, 1959.
15. Neuman, W. F. and M. W. Neuman. *Chemical Dynamics of Bone Mineral*. Chicago: Univ. Chicago Press, 1958, p. 115.
16. Ray, R. D., D. La Violette, H. D. Buckley and R. S. Mosiman. *J. Bone & Joint Surg.* 37-A: 143, 1955.
17. Schoenheimer, R. *The Dynamic State of Body Constituents*. Cambridge: Harvard, 1942.
18. Spencer, H., M. Li, J. Samachson and D. Laszlo. *Metabolism* 9: 916, 1960.

GENERAL DISCUSSION

Dr. Cotzias: There is no question that strontium can be used for comparative studies in bone disease. However, I do want to caution that it is not completely interchangeable with calcium as the work of Spencer, Lazlo, Comar, Catsch, etc. has shown.

Dr. Ray: It is well established that strontium and calcium are handled differently by the body. Strontium is excreted more rapidly in the urine (Comar, C. L., R. H. Wasserman, S. Ullberg and G. A. Andrews. *Proc. Soc. Exper. Biol. & Med.* 95: 386, 1957 and Spencer, H., M. Li, J. Samachson and D. Laszlo. *Metabolism* 9: 916, 1960). However, with double isotope studies Bauer and colleagues (Bauer, G. C. H., A. Carlsson and B. Lindquist. *Acta physiol. scandinav.* 35: 56, 1955), using Sr^{85} and Ca^{47}, showed that the calculated accretion rates are approximately the same for the two isotopes. There may be clinical conditions in which they are different, but this has not been demonstrated yet. There is one major clinical advantage in using Sr^{85} rather than Ca^{47}. Ca^{47} is a beta as well as a gamma emitter and as it is prepared today contains a certain amount of Ca^{45} another beta emitter. Bone-seeking beta emitters may cause damage to the marrow. Ca^{47}, since it has a short half-life, must be used at fairly high doses for adequate clinical studies extending over several weeks. To avoid this we have used Sr^{85}, a pure gamma emitter, with a half-life of 65 days.

Dr. Hastings: First I want to congratulate Dr. Ray very much for finding many answers to a question which those of us who were in calcium and bone research in the pre-isotope days simply couldn't do. I would like to ask one question and to make a short comment. The comment has to do with the remark that I believe you made, that calcium ion concentration in plasma was rather small. For the benefit of those who might wonder how small, I might say that it is roughly about half the total calcium concentration. The other half is reversibly combined with protein. The question has to do with my wondering whether or not the easily available calcium, easily exchangeable calcium in the bone pool, changes much with respect to age of the animals?

Dr. Ray: Yes, I think that's true. As the animal ages, the exchangeable pool may decrease. One of our problems is to reduce the figures to some physiological constant. Thus the figures are reported in plasma volumes and more recently we have attempted to use lean body mass.

Dr. Foreman: A possible explanation for the so-called non-exchangeable calcium in the dead bone might lie in the fact that dead bone develops a pannus after it is put into the body, either a growth of fibrous tissue in some cases or in rats, where studies are not done under sterile conditions, it will develop a pus coat. I think the nonexchangeability may be mechanical rather than a loss of kinetic exchangeability. It is actually a physical barrier.

Dr. Ray: I know my colleagues in the laboratory would resent your implication that the animals were infected. We do use a regular surgical sterile technique and any animals that become infected subsequently are discarded. It is possible that a physical barrier of fibrous tissue might develop between the bone and the surrounding tissues but the fact that the isotope is accreted with such amazing rapidity would indicate that there must be some interchange between the body fluids and the

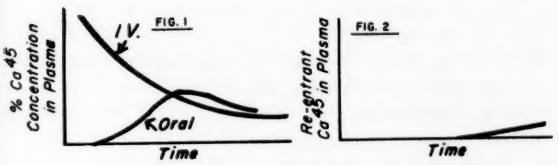


FIG. 1. Ca^{45} concentration in plasma after I.V. and oral administration.

FIG. 2. Re-entry of Ca^{45} into plasma.

bone. As time goes on, there is a tendency for the implanted devitalized bones to undergo resorption following vascularization. Thus, it would appear that accessibility of the dead bone to body fluids is increased with increasing time rather than decreased.

DR. HERTA SPENCER (Montefiore Hospital, New York): I would like to ask Dr. Ray to make some remarks in regard to the similarity of the accretion rates in two patients, the data of whom were shown in his first figure. One of the patients was 16 years old and the other 60 years, and still the accretion rates as well as the volume distribution of these two patients was similar, despite the marked age difference. One would assume that the accretion rate of younger persons is higher than that of the older persons, especially if the 60 year old has carcinoma of the thyroid with metastases to bone.

DR. RAY: This is one of the problems we encountered when we reduced the figures to lean body mass. The second reason may be, Dr. Spencer, that in the patient with thyroid metastases there was a more rapid turnover of bone than in a perfectly normal patient the same age. We are currently trying to increase our number of control patients so we can develop statistically valid data for various age groups.

DR. HIRAM HART (Montefiore Hospital, New York): I would like to address myself to several points in the preceding presentation. As to the general question of when a steady state is reached: First of all there is a steady state situation at the beginning, throughout the course of the experiment, and, finally, at the conclusion of the experiment the steady state continues. All that is being detected in these concentration curves as they rise and fall is the changing relative geographical distribution of the Ca^{45} and Sr^{85} isotopes. If we consider the original Ca^{45} to be introduced into the plasma or gastrointestinal tract, then following absorption the concentration of Ca^{45} will tend to go down, as indicated below (Fig. 1). If we assume that some of the Ca^{45} which has "entered" bone "returns" rather quickly, then after a while the concentration of the returning Ca^{45} fraction will start rising (Fig. 2). When you assay Ca^{45} concentrations in the plasma you may be simply measuring the sum of the falling curve of the initially injected material plus the rising contribution of re-entrant tracer calcium. It should not be surprising then that this sum flattens out at least until excretion overcomes the re-entrant contribution. The reciprocal of the Ca^{45} plasma concentration for the flat portion of the curve need not then necessarily represent a measure of the total exchangeable calcium and, as a matter of fact, there would not seem to be any clearly definitive evidence as yet that a truly discrete compartment of exchangeable calcium, as such, actually exists.

With regard to the complex 3, 4 or 5 compartment systems that have been proposed in various calcium metabolism studies, there are a few observations that can be made. The general theory of tracer analysis has been developed and it turns out that in evaluating a transport rate or volume you effectively have to calculate the quotient of two determinants. If you are dealing with three compartment systems you usually (though not always) end up with 3 by 3 determinants; four compartment systems give rise to 4 by 4 determinants, etc. If there is a 2 or 3 per cent error in one of the measurements it can be shown that the error introduced in these quotients may be high. It would seem of value then to consider the error matrix associated with a given system, that is, to determine the error introduced in a calculated parameter for certain amounts of error in the original measurements. Moreover, it would also seem worthwhile to indicate the extent to which the models proposed (however reasonable they may be) are uniquely determined by the experimental results.

The last point I would like to make is not specifically related to the work under discussion. In studying a multicompartment system one can, in principle, go a step further than determining the rates and volumes. A theory of perturbation-tracer analysis has been developed which may make possible determination of the functional dependence of these rates and volumes upon the concentrations within the compartments (Hart, H. *Bull. Math. Biophys.* 22: 41, 1960). The compartments can be either chemical or geographic and in principle the functional dependence of catalytic factors might be determined.

Summary remarks by the chairman

GEORGE C. COTZIAS

I AM CONVINCED that the Physiology session has made an excellent record which speaks for itself. Furthermore, all of us look forward to Dr. Calvin's concluding remarks, particularly since he has attended all the talks thus far and has taken copious notes as well. Therefore, instead of a summary, just a few words about future conferences.

Interdisciplinary meetings, such as this one, presuppose that men from one discipline will be useful to men in another. We are actually a very good example of this since even at this early stage many of the nonchemists have profited greatly from contact with the physical and theoretical chemists in our midst. An even higher degree of success, however, would be attained if the arrow were to point also in the other direction, namely, if we were to contribute something to them. Frankly,

I feel insecure on this point, one of the reasons being that, although we all need this relatively new breed of colleague, we often have a language barrier working against us when we try to communicate. It would be too obvious to say to a sophisticated audience that we in Biology and Medicine need to acquaint ourselves with the language of quantum mechanics. However, it might be less obvious to us that we also need to make ourselves intelligible to the others. As things seem to stand now, much of the insight regarding chelation reaching the biologist and the medical man is being gained by our colleagues while they are dealing with matters peculiar to their own areas, often without awareness of biological problems. Hence, the models which are being elaborated now might not fit the needs of biologists.

I wonder whether in future meetings it might not be desirable for our friends to lecture after they have heard the biologists out. Their dissertations will become even more helpful to us provided, of course, that we had made ourselves intelligible to them by avoiding some of our peculiar biological terminology. I have enough conceit as a biologist to say that if the problems of life were made intelligible a larger number of physical and theoretical chemists might find them fascinating and fertile.

Perhaps we should try a conference where life scientists will take extra pains to key their talks specifically to the highly sophisticated scientists among us who are laymen in biology. This type of thing might hasten the natural evolution of communication between our disciplines. In other words, it might work.

Known and suspected role of metal coordination in drug actions

MAYNARD B. CHENOWETH

Biochemical Research Laboratory, The Dow Chemical Company, Midland, Michigan

IN THIS PRESENTATION it is my aim to describe to the more chemically oriented participants some salient factors and theories relating to drug action, generally, and the action of metal-binding agents, particularly. Also, biologists who are not pharmacologists may not have thought of some of the special problems concerning the pharmacology of metal-binding drugs nor even those of pharmacology in general.

It is easy to divide the drugs themselves into two broad classes, each of which has some special problems more peculiar to itself than to the other class.

Class I describes those few agents which vigorously react with metals under nearly all biological conditions and which are not themselves acted upon by biochemical systems. Representative materials are ethylenedinitrilotetraacetic acid [the Chemical Abstracts nomenclature of the material more commonly known as ethylenediaminetetraacetic acid (EDTA)] and the fluoride ion. The fluoride ion could even be disqualified, for *Dichapetalum cymosum* must use it to make fluoroacetic acid (32). But, generally, there would appear to be very little these materials could do *in vivo* other than bind metals somewhat as they do *in vitro*. I say "appear" because even the simple CN^- ion can prove tricky, for its effect on ribonuclease is said to result from splitting an S-S link and has nothing to do with metals (17).

We may assume for the moment that these *Class I* ligands have no possibility of any mechanism of biological effect other than binding metals. Which metal is bound, where this occurs, and to what good or bad effect may not always be so easily decided, however.

When they are used *in vitro* you are on your own and *caveat emptor* is the rule for interpreting your data. However, in the total animal many more uncontrollable and unknowable factors intervene between administration and desired effect. When a desired result is at last obtained, it means that problems of stability, toxicity, absorption, distribution and excretion have been overcome and only unexpected toxic side effects remain to be considered. Such a situation is not unique to this class of drugs and is very familiar to drug company pharmacologists. Such phenomena are usually readily observed in laboratory animals prior to preliminary trial in humans, where they must be carefully taken into account.

Although the compounds of *Class I* may, perhaps, provide fewer acute technical difficulties than *Class II* drugs, this is more than compensated for by the addition of a new dimension: concern for the effects of long-term human exposure. This is notably true for both compounds chosen as representative. Aspects of this problem and of the therapeutic utility of such compounds will be better discussed by others, forcing me to struggle primarily with *Class II*.

Class II describes the enormous number of compounds which cannot be shown *not* to bind metals *in vivo*. Happily, there are a few drugs we can exclude, such as the inhalation anesthetics, xenon, cyclopropane and the like. But nearly all other drug actions *may* have metal-binding as the ultimate cause of their main or side action. It is a case of "guilty until proven innocent." Long contemplation of this problem clearly leads to madness.

There have been earlier reviews of this topic which attempted to organize the scanty and scattered data then available (9, 40). A report of a conference held last year is replete with observational details (33).

From all this background emerge several pitfalls and problems which I would like to discuss with you now.

1. Effect under observation may be fundamentally unrelated to metal-binding, despite overt evidence to the contrary. For example, 3,3′,5-triiodothyronine greatly increases the action of epinephrine *in vivo*. It also complexes metals and so potentiates epinephrine in *in vitro* tests (34). Does all this prove that either triiodothyronine or epinephrine exert important effects by reaction with a metal in the

biophase? Not at all. All this goes on in the dirty water of the isolated bath where epinephrine is oxidized to biologically inactive materials by the iron or copper usually present in electrolyte solutions. Addition of any of a wide spectrum of complexing agents may prevent this oxidation and so augment the action of a given concentration of epinephrine (9). I wonder how many of the elaborately described actions of γ-aminobutyric acid may be due to such mundane effect?

Anyone wishing to know if calcium activates an isolated enzyme system would probably add EDTA to complex the calcium. Peculiarities of the system enabled one investigator to detect the fact that EDTA was forming a complex with the acetate buffer and the carbohydrate substrate, producing a spurious effect not related to metals at all. (W. B. Neely, personal communication.)

From a clear water solution to the clinic is an immense leap, but it seems likely that the effectiveness of N-acetylpenicillamine in ameliorating certain pathological phenomena in Wilson's disease is unrelated to metal-binding or copper mobilization (20). This suggests that the mercapto function is concerned in some role other than metal-binding and that mono-mercaptans in general might be useful.

Bacq (2) has observed that many chelating agents protect against X-irradiation. On the other hand, it is his conclusion that they do not do so by trapping metals.

Although many drugs have functional groups which will coordinate with metals, one must caution against ascribing their *in vivo* action to metal-binding found *in vitro* until more proof is found for such an effect. Functional groups may actually have some other function.

2. *A living cell is not just a bag of salty water.* It is a rigidly prescribed lattice work or mosaic in which very few degrees of freedom are tolerated. In complex organisms cells and organs are very different in their chemistry, anatomy, physiology and pharmacology. Trite? Yes, but, although even a baby can differentiate a glass of water from its mother, a scientist is apt to become upset and puzzled when reactions occurring in the water are not found in the mother—or *vice versa.*

The hazards that confront a drug enroute through a whole organism to its ultimate site of action are so numerous that one wonders how any make it. Generally, undissociated, lipid-soluble molecules have the best chance and highly dissociated, water-soluble molecules, the least. Size *per se* is apparently not an important determinant, although it may dominate rate phenomena. The most toxic of all known molecules, botulinus toxin, is a large protein which escapes digestion by proteolytic enzymes and is absorbed sufficiently well from the gut. Polypeptides such as *Amanita phalloides* toxin also escape digestion and are well absorbed from the gastrointestinal tract. Chelating agents based on such polypeptides might be most interesting.

Avoiding the gut by intravenous injection is awkward and potentially more hazardous to the patient. Many blockades still await the molecule at interfaces, while secretory and excretory organs and catabolic enzymes abound. Simple dilution, too, is important, while concentration can occur in areas not in the experimenter's plan.

Above the intracellular complexities of this problem, there is what may be called the inter-organ level of complexity. For example, the beneficial effects of thiourea analogues on cardiovascular disease actually are mediated through depression of the thyroid gland and not at all directly upon the circulation. Although other examples probably exist in the metal-binding area, they have not occurred to me. This sort of interaction is found so frequently in other areas of biology as to warrant efforts to be sure a given effect is actually exerted primarily on the observed organ and not secondarily through an intermediary organ, which is the true primary target organ.

3. *Peculiar localizations of drugs are not much appreciated,* especially if they are in a tissue not primarily responsive to the drug. Such accumulations may result from compliance with basic laws regarding fat and water solubility or from active, energy-consuming transport either of the original molecule or its metabolic transformation products.

There is no reason to expect the highest concentration in the organ which it is desired to affect. While the liver is well known to contain more morphine than the brain and more digitalis than the heart, other organ avidities are known.

The adrenal is more avid than the brain for xenon (14) and several other anesthetics (10). None of these compounds are metabolized, so that only physical-chemical factors can be considered operative. The reported accumulation of lead in this gland (37) might, however, be either active or passive.

Fluoride was found in exceptionally high amounts in the pituitary of rabbits and cattle (13) and so was mercury in man (36). Adrenals and pituitary have been found to accumulate salicylamide (5) and aspirin (28). The thiocyanate ion even selects two out of the three anatomically differentiated salivary glands (12). It is difficult to guess whether all this might be active or passive.

Localized high concentrations of other less well understood complexing agents thus, no doubt, often occur. This may lead to a toxic or, less likely, to a desirable effect on a specific organ. Thus, EDTA selects body water and by virtue of the four carboxyl groups, finds it difficult to enter a lipid phase. One might guess in advance of Foreman's work that concentrations would be high in the kidney; a suspicion also enhanced by the known occurrence of renal pathology.

As to a possible mechanism for this toxicity, quite small doses are reported to decrease renal filtration by complexing iron ions said to regulate the diameter of renal vessels (38). However, this does not correlate well with the histologic changes in the tubules.

Metabolic transformation may alter distribution markedly (indeed, that is its usual "purpose"). Where the

metabolite is a metal-binding agent new effects may appear. Ring hydroxylation of 2-napthylamine renders it more water-soluble and capable of chelating. It is more than coincidence that this metabolite produces urinary bladder tumors (11). The kidney forms or contains exceptionally high concentrations of citric acid in fluoroacetate-treated animals (32), and it seems to me likely that the protection against lead demonstrated in these animals (16) is partially a result of the prompt escorting of lead citrate into the urinary phase.

On the other hand, an active molecule may be introduced into systems wearing a metabolizable disguise. For example, to detect β-glucuronidase, Fishman and Baker (15) introduced 8-hydroxyquinoline as the glucuronide and observed the freed base. Approaches of this sort have usually been unsuccessful when consciously made, perhaps because esterification was too often the mask employed.

Where a metal is known to occur in greater than average amounts it is to be expected that ligand molecules might accumulate. Dithizone attacks all organs rich in zinc, such as the eye, pancreas and prostate. It is interesting that the tetracyclines accumulate in bone to such an extent as to cause the bones to fluoresce under ultraviolet light, for they are avid for Ca^{++}.

The opposite kind of situation is seen with gluconic acid. Although this is a fair *in vitro* chelating agent, it is so briskly metabolized that effective concentrations probably never exist anywhere *in vivo*. Certainly, it seems to be nearly without pharmacology (8).

The other possible mechanism, active metabolic transport by some naturally existing system, may cause truly unpredictable accumulations. Clear-cut examples of transport of added metal-binding drugs are not available so far as I know, unless one may include Dr. Christensen's abnormal amino acid.

4. Role of weak bonds. The opinion is often voiced that a given drug does not act through metal-binding because its *in vitro* avidity for metals is so low. I cannot yet regard this as a valid objection.

Obviously, metabolic transport of metal ions through cell walls must require weak bonds which are readily reversible. Impairment of this function could be accomplished by only a slightly stronger bond to a new ligand between transport*er* and transport*ee*.

Weak coordinate bonds participate more readily in catalytic reactions for they are so easily reversible, while extremely strong bonds would not react any further. A metal ion such as Mg^{++} or Mn^{++}, which catalyzes an enzymatic system by forming loose complexes between substrate and enzyme, need not be absolutely locked up. Given the proper circumstances, merely hampering this function by a weak ligand foreign to the system could slow down some vital process, and the overall effect could ultimately become extreme. But then, why should not the more avid ligand be still more effective? It probably would be, were it to arrive in position still able to pick up this metal. However, the powerful ligand will surely be tightly loaded with metal long before, perhaps thus never reaching the critical site and, if it should reach it, find little incentive to drop iron or copper for magnesium or manganese.

It is well that this is a conceptual conference for proof of this idea is not easily obtained. No change in the quantity of metal present would be expected, and present techniques do not seem able to detect the metal's dissatisfaction with its working conditions. But even as in a factory, this state of affairs leads to lower production, perhaps as the only symptom!

This concept does suggest that one should not seek more generally powerful ligands, except perhaps in the case of removal of toxic metals and certainly when X-ray contrast agents are sought. Rather, extreme selectivity, even if of a much lower order of avidity, and employment of unique ligand groups are more likely to lead to new and useful drugs. As we stand today, most drugs known to function by coordinating with metals *in vivo* attack too many metals, while the more specific pharmacologic effects of other drugs are hard to pin down to metal coordination.

5. Relative availability of metals in vivo. A complexing agent can not exist uncomplexed in the presence of metals which it will bind. In biological systems there will always be ions, of which Ca^{++} will usually be the most accessible and in the most acutely critical balance. Unless one specifically desires to attack Ca^{++}, a ligand might best be introduced as the Ca^{++} complex. Intravenous injection, especially of any quantity of ligand, could otherwise lead to abrupt cardiovascular collapse. On the other hand, oral administration of the tetracyclines with excess of harmless aluminum or calcium salts is not recommended because there is a practical reduction in absorption.

In general, zinc seems to be the next most accessible divalent cation. According to Bartholomew *et al.* (3) about $\frac{4}{5}$ of the Zn^{++} of liver and tumors is freely available to EDTA. Overall urinary excretion of Zn^{++} in humans was increased enormously by $Na_2CaEDTA$ given intravenously (31). Zinc-rich organs are attacked by dithizone, and the pancreas is also affected by 8-hydroxyquinoline (21).

Other drugs which appear to act upon zinc-activated systems include phenylbutazone (39) and certain substituted dithiooxamides of considerable toxicity (25, 26). It seems highly probable that the various therapeutically important inhibitors of carbonic anhydrase act upon the enzyme's zinc content.

The relative overall availability of other physiologically important metals is probably Cu > Fe > Mn > Co, but anyone with a different and firm opinion will be deferred to. It is probable that the overall accessibility of a metal is not always the governing factor. The specificity of the ligand sometimes is capable of overriding excesses of more accessible ions. For example, various derivatives of phenothiazine form complexes with Mn^{++}. Only Fe^{+3} amongst the other cations of interest will react at all (4). If it can be shown that the activity of these drugs is related to this complex forma-

tion, it will become an exquisite example of specificity over-riding accessibility and excess.

Ultimately, we must come to grips with the problem of the large amounts of stray metal that is an "innocent bystander." Specificity of the ligand seems to be the only answer, even if it means polypeptides built of unnatural amino acids.

6. Effect of metals on natural ligands. Every coin has two sides, and we must consider that naturally occurring and vital ligands can be influenced in their work by excesses of normal metals or by abnormal metals. *A priori*, one would suppose the literature to be filled with details of this sort of action because one finds it hard to see many other ways in which a metal could be toxic. Perhaps I have looked in the wrong places, but I have found very few reports touching on the subject.

Thallium is said to react with histamine, perhaps accounting for its neurotoxicity (7). Histamine and beryllium form a complex (19) which is suggestively interesting because of the many roles attributed to histamine in the body. Amino acids leave the body with Y^{91} (22). Possibly this is true also with vanadium, as fingernail cystine content is reduced in human intoxication (27).

Some suspicion that chromium replaced the cobalt in cyanocobalamin to cause pernicious anemia in a case of chronic chromium poisoning has been offered (35). By complexing the substrate, titanium may interfere with tyrosinase (6). The hypothyroid effect of cobalt might be the result of complexing thyroxine (23).

A variant on this concept is possible. Rather than simple depletion of natural ligands, it is conceivable that a foreign metal-ligand complex is formed which is especially active as such. The mysterious toxic actions of beryllium particularly suggest this possibility.

7. Metal-ligand complex as the active agent. There are some examples of metal-ligand complexes which possess special pharmacological activities not seen with the individual components. The outstanding and familiar case is that described by Dr. Albert and his co-workers for 8-hydroxyquinoline and iron. Everything points to the 1:1 Fe-oxine complex as the active bactericidal material (1). This work merits careful study, but, as most of the participants are probably familiar with it and Dr. Albert is here, I will not detail it.

The cobalt in cobalamin may play a fundamental role of this nature as in the case of so many metalloenzymes. An example of the administration of preformed metal chelates, which may apply here, is the use of cobalt EDTA or cobalt histidine as an antidote for cyanide poisoning (24, 30). It is not certain whether Co^{++} ions are released to form $Co(CN)_2$ or whether the CN^- coordinates with the bound cobalt.

Lead ions can be activated by cysteamine in its effect upon isolated aorta strips. When added at a 10^{-4} M concentration alone neither lead acetate nor cysteamine had any effect, yet together a response was induced (18).

Doubtless many drugs act as a metal complex which attaches *in toto* to another system. As this complex presumably uses a biological metal, it provides a means whereby systems which are not metal-catalyzed may be inhibited by a metal-binding agent. Such a molecular species might well have specificities far more exquisite than those possessed by simple binding agents.

8. Importance of structure-activity relationships. Drug-induced shifts in metal levels in tissues, particularly of normal trace metals, are unusually difficult to measure with precision. Even if "$P < 0.01$," what does a 5 per cent change mean, anyway? Such an increase in copper content was found when we compared normal dog hearts with hearts from dogs dying of ouabain (a sterol cardiac glycoside) poisoning (29). Although this and other experiments merely suggest a direct influence of the digitaloids upon trace metal balance, my interest is kept up by reflecting upon the molecular variations, such as allomerization at C_{17}, which abolish their characteristic activity, for this also seems to abolish any potential metal chelation.

Better luck was had by Wiesel (41) who found that the various glucogenic corticosteroids react with Cu^+ in non-aqueous systems and that cortisone produces a quite marked elevation in kidney copper content. However, an analysis of structural variation among these compounds permitting or forbidding Cu^+ chelation and a correlation of this with hormonal activity might aid in deciding whether such a change is directly mediated by the cortisone *per se* or less directly by some effect induced by the drug. The fact that other complexing agents such as 8-hydroxyquinoline and the salicylates possess characteristic antiinflammatory power is exciting but not strictly pertinent to this argument. Certainly, variations in blood metal levels, especially of copper, are not likely to prove to be directly related to a drug action as it appears too many factors will influence them.

Determination of metal levels in tissues is likely to prove fruitless in most cases and difficult in all; controlled variation of metal levels is even more risky, while addition of preformed complexes is usually confusing. What approach is left? The most profitable has been the study of structure-activity relations so familiar to pharmacologists. In this case, instead of a nebulous "receptor," we have a metal. The structural changes which promote or retard metal-binding *in vitro* have something of a basis in measurable reality. However, the most elegant study of this sort, even taking into account metabolic, steric and other factors, leaves a sterile victory, for absolute proof cannot be obtained this way, either. One is left with data showing that only a compound capable of metal-binding *in vitro* is capable of exerting the biological effect, but no *evidence* that it exerts this effect by metal-binding. Logically rigid proof of anything biological is probably beyond our times. Even *cogito, ergo sum* is open to argument.

SUMMARY

A drug may be a metal-binding agent *per primum*, or be metabolized to a metal-binding agent. Even if the biological system under inspection is not metal depend-

ant in any sense, one can still picture a metal-drug combination attacking a key functional group. When such conclusions are seen against the background of knowledge of absorption, distribution and other factors common to all drug action theory, it begins to seem that a vast number of pharmacological effects are mediated through metal-binding. Proof for any one case will be hard won and only by a multi-disciplinary attack.

REFERENCES

1. Albert, A., A. Hampton, F. R. Selbie and R. D. Simon. *Brit. J. Exper. Path.* 35: 75, 1954.
2. Bacq, Z. M. *Actualités pharmacol.* 10: 5, 1957.
3. Bartholomew, M. E., R. Tupper and A. Wormall. *Biochem. J.* 73: 256, 1959.
4. Borg, D. C. and G. C. Cotzias. *Fed. Proc.* 19: 248, 1960.
5. Bousquet, W. F. and J. E. Christian. *J. Am. Pharm. A.* (Scient. Ed.) 49: 389, 1960.
6. Cavanaugh, D. J., J. Harris and J. Z. Hearon. *J. Am. Chem. Soc.* 77: 1531, 1955.
7. Champy, C. and S. Hatem. *C. R. Soc. Biol.* (Par) 151: 520, 1957.
8. Chenoweth, M. B., H. Civin, C. Salzman, M. Cohn and H. Gold. *J. Lab. & Clin. Med.* 26: 1574, 1941.
9. Chenoweth, M. B., *Pharmacol. Rev.* 8: 57, 1956.
10. Chenoweth, M. B., D. N. Robertson, R. Gohlke and D. S. Erley. *Pharmacologist*, Fall 1960.
11. Clayson, D. B. *Brit. J. Cancer* 7: 460, 1953.
12. Clemedson, J. C., B. Sörbo and S. Ullberg. *Acta physiol. scandinav.* 48: 382, 1960.
13. Demole, V., F. Fassa and E. Matthey. *Helvet. physiol. et pharmacol. acta.* 17: C 19, 1958.
14. Featherstone, R. M., W. Steinfeld, E. G. Gross and C. B. Pittinger. *J. Pharmacol. & Exper. Therap.* 106: 468, 1952.
15. Fishman, W. H. and J. R. Baker. *J. Histochem.* 4: 570, 1956.
16. Fried, J. F., M. W. Rosenthal and J. Schubert. *Proc. Soc. Exper. Biol. & Med.* 92: 331, 1956.
17. Gawron, O., J. Deil and A. J. Glaird III. *Biochim. et biophys. acta.* 19: 170, 1956.
18. Grévissé, J. and M. Goffart. *J. physiol., Paris* 51: 471, 1959.
19. S. Hatem. *Compt. rend. soc. biol.* 153: 574, 1959.
20. Hill, L. and J. M. Walshe. *Lancet* II: 444, 1959.
21. Kadota, I. and Y. Kawachi. *Proc. Soc. Exper. Biol. & Med.* 101: 365, 1959.
22. Kawin, B. *Arch. Biochem.* 45: 230, 1953.
23. Kriess, J. P. and W. H. Carnes. *Am. J. Med.* 17: 107, 1954.
24. Mercker, H. and G. Bastion. *Arch. exper. Path. u. Pharmakol.* 236: 449, 1959.
25. Miller, J. G., M. B. Chenoweth and T. M. Brody. *J. Pharmacol. Exp. Ther.* 121: 32, 1957.
26. Miller, J. G. and T. M. Brody. *J. Pharmacol. & Exper. Therap.* 121: 43, 1957.
27. Mountain, J. T., F. R. Stockell and H. E. Stockinger. *A.M.A. Arch. Indust. Health* 12: 494, 1955.
28. Pallot, G. and H. Eberhardt. *Compt. rend. soc. biol.* 150: 1220, 1956.
29. Palmer, J. F., D. R. Bennett and M. B. Chenoweth. *J. Pharmacol. & Exper. Therap.* 106: 409P, 1952.
30. Paulet, G. and R. LeBars. *J. physiol., Paris* 51: 547, 1959.
31. Perry, H. M. and E. F. Perry. *J. Clin. Invest.* 38: 1452, 1959.
32. Peters, R. A. *Advances Enzymol.* 18: 113, 1957.
33. Seven, M. J. (editor). *Metal-Binding in Medicine.* Philadelphia: Lippincott, 1960.
34. Shemano, I. and P. C. Fallon. *Proc. Soc. Exper. Biol. & Med.* 103: 282, 1960.
35. Stein, H. and L. Weissbecker. *Zentralbl. Arbeitsmed. u. Arbeitsschutz.* 3: 42, 1953.
36. Stock, A. *Biochem. Ztschr.* 304: 73, 1940.
37. Takaoka, K. *Folia Endocrinol. Japon* 30: 499, 1954.
38. Tregubenko, I. P. *Doklady Akad. Nauk S.S.S.R.* 110: 874, 1956.
39. Wallenfels, K. and H. Sund. *Arch. exper. Path. u. Pharmakol.* 232: 338, 1957.
40. Weinberg, E. D. *Bact. Rev.* 21: 46, 1957.
41. Weisel, L. L. *Metabolism* 8: 256, 1959.

DISCUSSION

Relations between zinc content and the selective cytotoxicity of diphenylthiocarbazone

FREDERICK S. PHILIPS

Sloan-Kettering Institute for Cancer Research and Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York City

Dr. chenoweth has effectively outlined the uncertainties associated with attempts to impute *in vivo* drug effects to metal-binding (3). It appears that we share the same skepticism regarding too facile extrapolation of test-tube reactions to *in vivo* phenomena. There are, nevertheless, a few agents having selective effects in the intact animal in which the causal involvement of metal-binding is a reasonable certainty. One such substance, already cited by Dr. Chenoweth, is diphenylthiocarbazone, the familiar metal reagent, dithizone. Although this substance has as yet no certain therapeutic utility, a study of its pharmacologic proper-

ties may serve as a relatively simple model for the kind of rigorous evidence sought by Dr. Chenoweth.

The specificity of action of dithizone may be a happy coincidence of optimum alignment among a number of independent factors. First, the agent's binding capacity is restricted to a limited number of metals of known biological essentiality: cobalt, copper, manganese, zinc, and iron. Secondly, in most tissues the concentration of zinc exceeds that of cobalt, copper, and manganese by at least one order of magnitude; indeed, one might even question the biological classification of zinc as a "trace" metal. Thirdly, certain tissues, such as the mature prostate and the *tapetum lucidum* of the eye of carnivores, contain especially abundant accumulations of zinc. Fourthly, the physical and chemical properties of dithizone and of its metal complexes could be expected to contribute to its unique biological effects. On the one hand, at physiological hydrion concentration it is largely anionic with high aqueous solubility favoring rapid dispersal throughout the extracellular compartment. On the other hand, the magnitude of the oil-water partition coefficient of the undissociated molecule would tend to promote rapid entrance into intracellular spaces. Its complexes with metals are stable at the pH of the cell interior. Finally, there are additional factors of a biochemical nature which may also be involved in its specificity of action. After parenteral injection dithizone is firmly bound in plasma, presumably to proteins, to the extent that it cannot be detected in ultrafiltrates prepared by centrifugation through cellophane. Only traces are excreted in urine. Nevertheless, it is rapidly metabolized *in vivo* with the result that significant concentrations are found in most tissues for only brief periods (17).

Dithizone has two outstanding effects. High doses are convulsant and likely to be fatal within a few minutes (7). This acute toxicity is presumably due to disturbances within the central nervous system of which more will be said below. Doses less than acutely lethal are cytotoxic to a select group of tissues: namely, the insulin-producing cells of the pancreatic islets, the retina and choroid in the eyes of certain mammals, and the prostate gland.

Destruction by dithizone of the beta cells of the pancreatic islets resulting in experimental diabetes mellitus was first demonstrated in rabbits by Kadota (10). The use of dithizone for this purpose was prompted by the belief that the beta cells contain an abundance of zinc essential in the biosynthesis of insulin. Kadota surmised that the binding of the metal by a foreign agent would be intolerable to the cell. The rationale has no basis in fact since, as Vallee has stated, direct determination of the zinc content of pancreatic islets has not yet been feasible (18). Nevertheless, Kadota's observation and the later demonstrations of the localization of pink color in the islets of the rabbit pancreas, by intravital or histochemical staining with dithizone, are indications for the presence of some metal in abundance (12, 22).

The optic effects of dithizone have been studied in connection with investigations of the zinc content of the retina and choroid of the eyes of carnivores in which the metal is present in amounts exceeding, by several orders of magnitude, that found in most mammalian tissues (18, 20). The bulk of zinc is localized in the *tapetum lucidum* (21), a layer of specialized choroid cells which are responsible for the brilliant mirror-like reflectivity that is so characteristic of the carnivore eye. The reflecting capacity is due to intracellular, crystalline deposits of a cysteine complex of zinc which has been isolated from the tapetal region in good yield (19). The crystals may be part of the orderly array of intracellular rod-like bodies which have recently been visualized in electron microscopic studies of the cat tapetum (1). Within a matter of minutes after intravenous injection of dithizone into dogs or cats, a pink stain develops in the eyegrounds, presumably due to the formation of zinc dithizonate (5, 21). In time, edema and hemorrhage become prominent in the tapetal region of the choroid which may advance sufficiently to produce retinal detachment and blindness within 24 to 48 hours (5, 21).

While the unique structure of the *tapetum lucidum* appears closely related to the susceptibility of the carnivore eye, there is no comparable explanation for the retinal damage caused by dithizone in rabbits (2). Nevertheless, among noncarnivores this species is exceptional for the high concentration of zinc found in its eye coats (20).

The prostate gland of sexually mature mammals is differentiated for the accumulation of relatively high concentrations of zinc (9, 14–16). On this basis and with the encouragement of Kadota's earlier findings in the rabbit pancreas, my colleagues, Lo, Hall, and Whitmore, reasoned that a powerful complexing agent might have selective effects *in vivo* on prostatic tissue. Their eventual aim was a possible chemotherapeutic attack on human prostatic carcinoma which in some instances may retain the specific capacity for zinc concentration that is shown by the normal organ. They were successful in demonstrating dithizone's capacity to interfere with prostatic secretions in the dog and the effect was associated with extensive and long-lasting pathological alteration of the gland (11). Subsequently, we were able to generalize dithizone's capacity for selective damage in the mammalian prostate by demonstrating the effect in rats, rabbits, and rhesus monkeys.[1]

The study of the rat prostate has been particularly instructive. In this species the gland is made up of ventral and dorsolateral lobes which are anatomically separable and distinguished by contrasts in zinc metabolism. The dorsolateral lobe and especially its lateral portion have an abundance of zinc while the ventral lobe's accumulation of the metal does not differ from that of most mammalian tissues (8, 9, 14–16). Shortly after intravenous injection of dithizone, the dorsolateral lobe is stained pink or red, and the stain is particularly

[1] Unpublished observations of S. S. Sternberg, T. Hall, H. S. Schwartz, W. F. Whitmore, Jr., and F. S. Philips.

intense in the lateral portion. There is little or no stain in the ventral lobe (4). Quantitative measurement of dithizone in tissues confirms the visual impression.[1] The dorsolateral prostate accumulates and retains the agent in concentrations above that in blood. In the ventral prostate and in most other tissues the concentration of dithizone is below that found in the circulation and it falls rapidly. (Liver is exceptional in its relatively prolonged retention of low concentrations.)

Carbon tetrachloride extracts of the colored dorsolateral lobe have an absorption spectrum identical with that of zinc dithizonate. Washing the extracts with dilute acid converts the spectrum to that of dithizone. Analysis reveals relatively constant molar proportions of zinc and dithizone in the uncolored ventral lobe, in the deeply pigmented lateral portion of the dorsolateral lobe, and in the less colored dorsal region of the dorsolateral lobe. For example, 30 minutes after doses of 50 mg/kg the zinc-dithizone molar ratios are 8.5 in the ventral lobe, 5.9 in the dorsal, and 5.1 in the lateral. By contrast the dithizone content (in mg/gm dry weight) varies more than fifty-fold: ventral, 0.03 < dorsal, 0.32 < lateral, 1.75. The localization of pathological change in the rat prostate corresponds closely with the differences in zinc distribution and dithizone uptake: the ventral lobe is not affected; the dorsal region shows scattered foci of distinct, yet moderate, alteration; the lateral region is uniformly and severely injured (17).

It seems reasonable to conclude that the localization of dithizone and its effects in the *tapetum lucidum* of the eyes of carnivores and in the mammalian prostate are due to the large quantities of zinc that are available for complex formation. We have found that other metal-binding agents can also produce prostatic damage. Among these are 8-hydroxyquinoline, ethyl and isopropyl xanthate, and 1-(β-mercaptoethyl)-pyridine.[1] The localization explains only the selectivity of action. The mechanism of the cellular disturbance caused by large amounts of intracellular dithizone (or zinc dithizonate) is unknown. We anticipate that a number of metalloenzymes will be found to be affected in prostatic tissue; yet it may not be an easy task to determine which, if any, of these effects are the primary cause of cell pathology.

One final phenomenon merits attention. Shortly after the intravenous injection of dithizone, a remarkable localization of pink staining is found in a few sharply defined regions of the mammalian brain. Of particular note is the deep pigmentation of portions of the hippocampus (6, 13). It will be of interest to learn whether this pattern is directly concerned with the acute convulsant properties of dithizone and with the special behavioral changes which have been described in cats. Almost certainly there must be involved a unique accumulation of a dithizone-complexing metal (or metals). Zinc has been suggested in a preliminary study (13).

REFERENCES

1. Bernstein, M. H. and D. C. Pease. *J. Biophys. & Biochem. Cytol.* 5: 35, 1959.
2. Butturini, U., A. Grignolo and A. Baronchelli. *Gior. clin. med.* 34: 1253, 1953.
3. Chenoweth, M. B. This symposium.
4. Fleischhauer, K. *Naturwissenschaften* 44: 589, 1957.
5. Fleischhauer, K. *Verh. anat. Ges. auf der 55.* Versammlung (9–13 April), 69, 1958.
6. Fleischhauer, K. and E. Horstmann. *Z. Zellforsch. u. mikroskop. Anat.* 46: 598, 1957.
7. Fleischhauer, K. and F.-K. Ohnesorge. *Arch. exper. Path. u. Pharmakol.* 235: 63, 1958.
8. Gunn, S. A. and T. C. Gould. *Proc. Soc. Exper. Biol. & Med.* 92: 17, 1956.
9. Gunn, S. A., T. C. Gould, S. S. Ginori and J. G. Morse. *Proc. Soc. Exp. Biol. & Med.* 88: 556, 1955.
10. Kadota, I. *J. Lab. & Clin. Med.* 35: 568, 1950.
11. Lo, M.-C., T. Hall and W. F. Whitmore, Jr. *Cancer* 13: 401, 1960.
12. McNary, W. F., Jr. *J. Histochem.* 2: 185, 1954.
13. Maske, H. *Naturwissenschaften* 42: 424, 1955.
14. Mawson, C. A. and M. I. Fischer. *Nature, London* 167: 859, 1951.
15. Mawson, C. A. and M. I. Fischer. *Arch. Biochem. Biophys.* 36: 485, 1952.
16. Mawson, C. A. and M. I. Fischer. *Canad. J. M. Sc.* 30: 336, 1952.
17. Schwartz, H. S., T. Hall, S. S. Sternberg and F. S. Philips. *The Pharmacologist* 2: 83, 1960.
18. Vallee, B. L. *Physiol. Rev.* 39: 443, 1959.
19. Weitzel, G., E. Buddecke, A.-M. Fretzdorff, F.-J. Strecker and U. Roester. *Hoppe-Seyler's Ztschr. physiol. Chem.* 299: 193, 1955.
20. Weitzel, G. and A.-M. Fretzdorff. *Hoppe-Seyler's Ztschr. physiol. Chem.* 292: 221, 1953.
21. Weitzel, G., F.-J. Strecker, U. Roester, E. Buddecke and A.-M. Fretzdorff. *Hoppe-Seyler's Ztschr. physiol. Chem.* 296: 19, 1954.
22. Wolff, H., H. Maske, B. Stempfl and F. Baumgarten. *Arch. exper. Path. u. Pharmakol.* 216: 440, 1952.

[1] Unpublished observations of S. S. Sternberg, T. Hall, H. S. Schwartz, W. F. Whitmore, Jr., and F. S. Philips.

DISCUSSION

Known and suspected role of metal coordination in actions of antimicrobial drugs[1]

EUGENE D. WEINBERG

Department of Bacteriology, Indiana University, Bloomington, Indiana

DR. CHENOWETH HAS INDICATED that our suspicions far outweigh our knowledge concerning the role of metal coordination in drug actions; and this is certainly true with respect to antimicrobial drugs. My discussion of his paper will consist mainly of the listing of various theories (suspicions) plus some fragmentary evidence that has given rise to the theories. I hope that this discussion will help emphasize the great need for more intensive investigation of a number of interesting but isolated observations.

As is true of many classes of chemotherapeutic substances, a large majority of the presently known antimicrobial compounds *a*) possess theoretical metal-binding sites, *b*) have been demonstrated to be moderate or strong metal-binding agents, and/or *c*) are affected by specific trace metals associated with the biological system under consideration (1, 34, 37). The consequences of the possession of metal-binding activity by antimicrobial compounds can be divided into two kinds: those that are concerned with the host and those concerned with the microbes.

METAL-BINDING ANTIMICROBIAL DRUGS IN RELATION TO THE HOST

One of the usual requisites of an antimicrobial drug is that it should neither affect nor be affected by the host. Nevertheless, the introduction into the host of antimicrobial compounds that possess strong metal-binding activity may sometimes have one of the following consequences.

First, unusually high concentrations of a specific trace metal in a localized area of the host may interfere with diffusion through the tissues or with the antimicrobial activity of the drug. For example, the assimilation of the tetracyclines from the intestinal tract is suppressed by iron and calcium in the diet and is enhanced by such ligands as citrate and metaphosphate (7); and the efficacy of these antibiotics in the treatment of mastitis is depressed by the high calcium content of the affected tissues (28). Dr. Chenoweth has mentioned the accumulation of the tetracyclines in bone because of their avidity for calcium.

Second, unusually high concentrations of a specific antimicrobial drug may adversely affect metal activated host processes. Examples include *a*) the suppression by the tetracyclines of magnesium activated fatty acid oxidation in liver cells, of calcium activated blood coagulation, and of calcium activated cardiac function (34); and *b*) the initiation of the diabetic syndrome by a drug of the thiosemicarbazone series which presumably acts by combining with pancreatic zinc (3, 11).

Third, very low concentrations of metal-binding antimicrobial drugs may accelerate significantly the rate of growth of young plants, birds, and mammals. This action of the drugs obviously is not associated with the intestinal or other contaminating bacterial flora for three reasons: *a*) growth of germ-free plants and birds is stimulated by the compounds (25, 26), *b*) growth of conventional mammals is stimulated by parenteral injection of the antibiotics (8, 31), and *c*) a strong antimicrobial drug such as chloramphenicol (which does not bind metals) does not stimulate growth. Young plants, birds, and mammals require comparatively large amounts of such trace metals as zinc, manganese, and cobalt for rapid and efficient growth; and the theory has been advanced that such metal-binding agents as bacitracin, the tetracyclines, and streptomycin are acting as carriers of scarce quantities of one of these metals (37). The tetracyclines, for example, have been found to promote the absorption or the utilization of manganese by chicks (27); and a new organic growth factor has been discovered which replaces or renders zinc more available for chicks (6). The use of iron chelates of EDTA to promote the uptake of iron by plants grown in iron deficient soils is well established (3) and several other reports of the stimulation of growth by metal-binding agents have been published (20, 24, 33). The assumption is made that growth stimulation of either germ-free or conventional animals by antibiotics does not occur if optimum kinds and concentrations of trace metals are included in the diet. To test this assumption, it is recommended that in subsequent experiments on

[1] It is a pleasure to acknowledge financial support by the Public Health Service (Grant E-2252) and by Commercial Solvents Corporation of the experimental work performed in the author's laboratory on these problems.

the role of dietary antibiotics, controlled variations be made in the metal nutrition of the animals.

METAL-BINDING ANTIMICROBIAL DRUGS IN RELATION TO THE MICROBE

It is becoming increasingly apparent that each metal-binding antimicrobial drug requires a specific metal for maximum biological activity. The biochemical mechanism underlying the requirement in each case has not been elucidated, as yet, but several theories have been proposed and some experimental evidence for the theories has been obtained. The theories include the following:

1) *Metal is required for stability of the drug molecule.* Many protein molecules require a specific metal for the maintenance of a biologically active structural configuration and it is logical to expect that the peptide antibiotics would likewise possess such a requirement. Of the peptide antibiotics, only bacitracin has been studied in this respect and it has been found to require one atom of zinc per drug molecule for maximum thermal and storage stability (16). Zinc cannot be replaced by any other metal. Bacteriostatic and bacteriolytic activities of bacitracin (tested by procedures which require more than five hours for completion) are enhanced very markedly by zinc (36) whereas suppression by the drug of protein synthesis of resting cells (tested by a procedure that requires not more than five hours for completion) is enhanced only slightly by zinc (unpublished observation of Dr. J. L. Smith). Other trace metals are inactive in either type of test. It is possible, of course, that the enhancement by zinc of the bacteriostatic and bacteriolytic activities of bacitracin is associated in part with one of the mechanisms listed below as well as with the maintenance of a biologically active configuration of the peptide molecules for long periods of time.

2) *Metal is required for entry of the drug molecule into the microbial cell.* The requirement for iron for the antimicrobial action of 8-hydroxyquinoline is possibly due in part to the fact that the saturated (3:1-) complex is capable of penetrating the cell whereas the unsaturated (2:1- and 1:1-) iron complexes probably are prevented from entering (1). The observation that the metal chelates of *p*-aminosalicylic acid and isoniazid are more antitubercular than the nonchelated drugs may be associated with the fact that the chelates are more fat soluble and thus may be more capable of penetrating the intact cell (12).

3) *Metal is transported into the microbial cell by the drug molecule.* Dr. Albert has summarized many of the known examples of the ability of a ligand to increase the biological activity of a metal (1). In at least some instances, the ligand might merely serve to carry the metal into the cell and the resulting increased concentration of the metal might be the actual toxic entity. In other instances, the ligand might further increase the toxicity of the metal by altering its electron distribution or oxidation-reduction potential (2). Dr. Foye has postulated that the antibacterial action of a series of chelates of amino acids, peptides, and biotin is caused by the fat solubilization of the metals which then become toxic at the cell surface or within the cell itself (4).

4) *Metal is required for a secondary manifestation of the action of the drug molecule.* Autolytic enzymes of gram-positive bacteria are metal activated and lysis is one of the visible results of the antibiotic action of penicillin. Dr. J. L. Smith and I have observed that selected concentrations of EDTA permit penicillin to exert its primary antibacterial action but prevent lysis from occurring. Perhaps EDTA is merely suppressing the cells' lytic mechanism that is activated by the initial penicillin injury but further investigation of this observation is needed before a definite hypothesis can be advanced. In contrast to penicillin, the antibacterial (and consequently the lytic) action of bacitracin is suppressed by EDTA which is probably binding the zinc essential for stability of the drug, whereas neither the antibacterial nor the subsequent lytic effect of cycloserine is affected by EDTA. Apparently the initial biochemical lesion produced by cycloserine results in a greater amount of cellular disorganization than that produced by penicillin.

5) *Metal is required for inactivation of a metabolite or of an enzyme by the drug molecule.* The iron and copper complexes of diethyldithiocarbamate are stronger oxidizing agents than the free metal ions (the ligand by itself is not toxic) and have been postulated to act by destroying thioctic acid (2). The magnesium complexes of the tetracyclines have been found to inhibit a purified soluble alanine dehydrogenase extracted from tubercle bacilli whereas the noncomplexed drugs were inactive (14). However, the analogous enzyme extracted from a different genus of bacteria was not sensitive to either the pure or magnesium-complexed drugs (14).

It is obvious that if a drug molecule requires a specific metal and a metal-binding site for biological activity, the alteration or removal of such a site will depress or eliminate activity. Several studies have indicated that alteration does have this effect (1, 34). On the other hand, chemical modifications of drug molecules to promote or intensify metal-binding have not as yet yielded compounds with stronger antimicrobial action than the parent substances (34). Although the ability to complex metals appears to be essential for the biological activities of the majority of antimicrobial compounds, other factors associated with their molecular architecture are evidently of overriding importance. These factors are to be discussed by Drs. Albert and Foye (2, 12).

A consequence of the metal-binding activity of antimicrobial drugs that is important not to the host or the microbe within the host but rather to the microbiologist and the microbes in his laboratory is the fact that *in vitro* assay of some of the drugs is affected by the composition of the culture medium (35). The media in current use often contain large excess quantities of such metals as calcium, magnesium, iron, and manganese. Depending on the quantity and proportion of each

of these metals and/or the kinds and amounts of natural ligands in the medium, the antimicrobial action of some of the drugs may be considerably enhanced or depressed. Unfortunately, the producers of processed culture media can supply very little or no information concerning the concentration of metals in their products and no information whatsoever as to the extent of the constancy of metal contamination in successive batches of the same product.

Several recent reports have described the conversion of such amino acids as serine, cysteine, and methionine and such vitamins as riboflavin and biotin from growth factors to growth inhibitors by formation of their cobalt, copper, or manganese complexes (4, 29, 38). As stated previously, Dr. Foye has suggested that, in the complexed state, the metal becomes lipid soluble and thus can be carried into sensitive areas of the cell. Might it not be also possible that the metal remains attached to the growth factor and prevents a more physiologically normal metal from being complexed by the factor? The abnormal metal-factor complex might not be able to function properly in the subsequent incorporation of the factor in a peptide chain or in a coenzyme system and would thus poison the cell.

The final item I wish to discuss does not concern the role of metal coordination in the action of antimicrobial drugs but rather the role of metals in the microbial biosynthesis of the natural products among these compounds. Whenever a distortion of cellular metabolism is desired so that an unusually large quantity of a metabolite is to be produced, a shift in the quantities of one or a few trace metals from those concentrations needed merely for good growth is required. Such a shift, for example, is needed for the production in large quantity of such metabolites as citric (22) and fumaric (30) acids, riboflavin (15) and vitamin B_{12} (17), and diphtheria toxin (10). Among the antibiotics that have been most thoroughly studied in this regard, added manganese is needed for production of bacitracin (19); added zinc, iron, and calcium for actinomycin (21); added iron and subtracted copper for penicillin (23); added iron for prodigiosin (18) and pyocyanine (13); added calcium or magnesium for the tetracyclines (9); and added calcium or subtracted iron for streptomycin (32).

Among the possible mechanisms of action of specific trace metal ions in promoting or depressing a biosynthetic process are the following:

1) the metal may be required as a component of the metabolite; examples include cobalt in vitamin B_{12}, calcium in amylase of *Bacillus subtilis*, and possibly iron in prodigiosin;

2) the metal may be required for one of the enzymes in the biosynthetic pathway; an example is molybdenum in nitrogen fixation;

3) the metal may enhance autolytic enzymes so that more of the metabolite is released into the culture filtrate; a possible example is the stimulatory action of manganese in the bacitracin fermentation;

4) the metal may neutralize the antagonistic action of the metabolite toward the producing microbe; an example is the use of calcium or magnesium in the tetracycline fermentation;

5) the metal may antagonize a metal that activates a metabolite-destroying enzyme; an example is the use of calcium to antagonize iron in the streptomycin fermentation; and

6) the metal may activate a metabolite-destroying enzyme; examples include iron in the streptomycin fermentation and possibly manganese in the bacitracin fermentation.

Undoubtedly the quantitative trace metal requirements for specific biosyntheses by plant and animal cells may, as with microbial cells, differ considerably from the metal requirements for cellular multiplication. Dr. Curran, for example, has in this symposium discussed the effect of manganese, vanadium, and chromium on cholesterol biosynthesis (5). Research in this area of trace metal biology will certainly increase our understanding of many aspects of molecular biology and cellular physiology.

SUMMARY

A large majority of the presently known antimicrobial compounds are metal-binding agents. The metal-binding property of the compounds accounts for *a*) some of the problems associated with their diffusion through host tissues, *b*) many of their beneficial and harmful side effects on the host, and *c*) some of the discrepancies encountered in their *in vitro* assay. The compounds usually require appropriate concentrations of specific trace metals for maximum biological activity and it is probable but not certain that the actual antimicrobial entities are the unsaturated metal-drug complexes. Microbial growth factors can, under some conditions, be converted to microbial growth inhibitors by forming complexes with trace metals. The microbial biosynthesis of unusually large quantities of antibiotics, as well as other natural products, invariably requires a shift in the dietary concentrations of one or a few specific trace metals from the quantities required for normal cellular multiplication.

REFERENCES

1. Albert, A. In: *The Strategy of Chemotherapy*, edited by S. T. Cowan and E. Rowatt. London: Cambridge, 1958, p. 112.
2. Albert, A. *Fed. Proc.* This Symposium.
3. Chenoweth, M. B. *Pharmacol. Rev.* 8: 57, 1956.
4. Counter, F. T., Jr., R. N. Duvall, W. O. Foye and R. W. Vanderwyk. *J. Am. Pharm. A.* (Scient. Ed.) 49: 140, 1960.
5. Curran, G. L. *Fed. Proc.* This Symposium.
6. Dam, R., A. B. Morrison and L. C. Norris. *J. Nutrition* 69: 277, 1959.
7. Dearborn, E. H., J. T. Litchfield, Jr., H. J. Eisner, J. J. Corbett and C. W. Dunnett. *Antibiotic Med.* 4: 627, 1957.
8. Dick, E. C. and K. R. Johansson. *Antibiotics & Chemother.* 7: 349, 1957.

9. DiMarco, A. and P. Pennella. *Prog. Indust. Microbiol.* 1: 47, 1959.
10. Drew, R. M. and J. H. Mueller. *J. Bact.* 62: 549; 1951.
11. Escovitz, W. E. *Am. Rev. Tuberc.* 66: 373, 1952.
12. Foye, W. O. *Fed. Proc.* This Symposium.
13. Frank, L. H. and R. D. DeMoss. *J. Bact.* 77: 776, 1959.
14. Goldman, D. S. *J. Biol. Chem.* 235: 616, 1960.
15. Goodwin, T. W. *Prog. Indust. Microbiol.* 1: 137, 1959.
16. Gross, H. M. *Drug and Cosmetic Ind.* 75: 612, 1954.
17. Hall, H. H., R. G. Benedict, C. F. Wiesen, C. E. Smith and R. W. Jackson. *Appl. Microbiol.* 1: 124, 1953.
18. Harned, R. L. *Appl. Microbiol.* 2: 365, 1954.
19. Hendlin, D. *Arch. Biochem.* 24: 435, 1949.
20. Jordan, D. C. *Bact. Proc.* 1959, p. 41.
21. Katz, E., P. Pienta and A. Sinak. *Appl. Microbiol.* 6: 236, 1958.
22. Kitos, P. A., J. J. R. Campbell and N. Tomlinson. *Appl. Microbiol.* 1: 156, 1953.
23. Koffler, H., S. G. Knight and W. C. Frazier. *J. Bact.* 53: 115, 1947.
24. Lankford, C. E., T. Y. Kustoff and T. P. Sergeant. *Bact: Proc.* 1957, p. 55.
25. Luckey, T. D. In: *Proceedings of First International Conference on Use of Antibiotics in Agriculture*, Pub. 397, Nat. Acad. Sci., Nat. Res. Council, 1956, p. 135.
26. Nickell, L. G. and A. C. Finlay. *J. Agr. Food Chem.* 2: 178, 1954.
27. Pepper, W. F., S. J. Slinger and I. Motzok. *Poultry Sci.* 32: 656, 1953.
28. Price, K. E., Z. Zolli, Jr., J. C. Atkinson and H. G. Luther. *Antibiotics & Chemother.* 7: 672, 1957.
29. Ramsey, H. H. *Antonie van Leeuwenhoek* 23: 226, 1957.
30. Rhodes, R. A., A. J. Moyer, M. L. Smith and S. E. Kelley. *Appl. Microbiol.* 7: 74, 1959.
31. Rusoff, L. L., J. M. Fussell, C. E. Hyde, R. M. Crown and L. S. Gall. *J. Dairy Sci.* 37: 488, 1954.
32. Shaw, R. K., R. D. Henderson and W. J. Seagers. *Appl. Microbiol.* 8: 12, 1960.
33. Weinberg, E. D. *Bact. Proc.* 1957, p. 54.
34. Weinberg, E. D. *Bact. Rev.* 21: 46, 1957.
35. Weinberg, E. D. *Am. J. Clin. Path.* 30: 344, 1958.
36. Weinberg, E. D. *Antibiotics Ann.* 1958–59, p. 924.
37. Weinberg, E. D. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 329.
38. Weinberg, E. D. *Antonie van Leeuwenhoek* 25: In press, 1960.

GENERAL DISCUSSION

Dr. Thomas H. Jukes (American Cyanamid Company, New York): I should like to ask Dr. Weinberg what experimental evidence there is for the conclusion that antibiotics promote the growth of germ-free animals. I have never seen evidence along these lines. All the published investigations with which I am familiar show that in the case of germ-free animals there is no growth promoting effect of any of the antibiotics. Furthermore, the growth promoting effects of antibiotics injected into conventional animals can be explained by the known excretion of the antibiotics in question in the bile, so that they get back into the intestine where they exert their familiar effects on the intestinal microflora. The lack of growth promotion by chloromycetin may have various explanations including that of its absorption from the stomach, but it should be borne in mind that there are certain antibiotics that do not exert the growth promoting effect. This is not necessarily due to any metal involvement but could be due to their lack of specific action against those intestinal microflora that depress the growth of normal animals.

Dr. Weinberg: With respect to growth promotion of plants, this was worked on by Louis Nickell at Pfizer (Nickell, L. G. and A. C. Finlay. *J. Agr. Food Chem.* 2: 178, 1954) who showed that bacitracin, penicillin, streptomycin, and the tetracyclines but not chloramphenicol do stimulate growth of germ-free plants. With respect to stimulation of germ-free animals I agree with Dr. Jukes that there is a split in the literature. Dr. Luckey has a good survey of this in the book entitled *Antibiotics* (Goldberg, H. S., (editor). *Antibiotics*, Princeton: van Nostrand, 1959, p. 223). Dr. Luckey has demonstrated that low level feeding of terramycin—that is less than ten micrograms per milliliter of food—does stimulate the rate of growth of germ-free chicks.

Dr. Jukes: I am familiar with that argument and I regard it as unsound, the effects are hand-picked. He has omitted some data published elsewhere. Sometimes he uses only one animal per group and he has stated that, at low levels, oxytetracycline promoted growth in germ-free animals but at 50 ppm it depressed growth. Now it is well known that levels up to 5,000 ppm of oxytetracycline do not depress growth in conventional animals. In spite of that, Dr. Luckey argues that 50 ppm should depress growth in germ-free animals which seems to me to have no basis. Furthermore, Forbes' much more careful work with germ-free animals indicated that antibiotics do not produce either an increase or a depression in growth.

Dr. Klaus Schwarz: Through proximity I am familiar with the more recent work, and I wish to second entirely what Dr. Jukes has said. In all well controlled studies of which I know a beneficial effect of antibiotics on germ-free animals has not been demonstrable.

Dr. Weinberg: I would like to say that it is obvious that the balance of metals in the environment must be controlled very rigidly. In other words, if you have enough metals in the diet of the germ-free animal, then naturally you are not going to see any stimulation by antibiotics. If you make the diet deficient as Nickell did with his plants, then it is possible that you might see some stimulation. Pepper, for example, obtained stimulation with conventional animals fed a manganese-deficient diet plus chlortetracycline but no stimulation was obtained on a diet with sufficient manganese (Pepper, W. F., S. J. Slinger and I. Metzer. *Poultry Sci.* 32: 656, 1953). Now the germ-free investigators usually have a lot of metals in the diets of their animals and, therefore, many of their studies yield negative results.

Dr. Williams: I should like to make a statement in reference to Dr. Philips' paper. The stability of dithizonate ion complexes is in the order $Mn^{++} < Fe^{++} < Co^{++} < Ni^{++} < Zn^{++} < Cu^{++}$. Here is another example of the high affinity of copper and zinc for sulfur ligands. In discussing effects of dithizone, the lower levels of copper than zinc must not be allowed to obscure the greater affinity of copper for this ligand. Elsewhere we will discuss the properties of copper-sulfur binding.

Dr. Philips: Dr. Williams' cautions are most welcome for I have carefully avoided the inference that intracellular binding of zinc is the mechanism of action of dithizone. We do believe our work and that of others permits only the reasonable deduction that selective tissue susceptibility is correlated with unusually high zinc content. The most compelling evidence is the difference in response among the various regions of rat prostatic

tissues where zinc content, dithizone uptake, and pathology have been directly related. It also could be that the binding of the metal in zinc-rich tissues is the direct cause of pathology; but there are alternative possibilities. Conceivably, in zinc-rich cells the relatively large amounts of zinc dithizonate formed could act as an intracellular depot providing by mass action a prolonged, steady concentration of free dithizone. By such a buffering mechanism susceptible metalloenzymes, whether they be zinc or copper dependent, might remain inhibited sufficiently long to become irreversibly inactivated or to cause irreversible changes in other metabolic systems dependent on these enzymes. In cells less rich in zinc, where the buffering effect of a depot of the dithizonate would be less prominent or even absent, inhibitory concentrations of dithizone would exist for times too brief to cause irreversible effects. The degradation of dithizone by tissues could also be a critical factor in the duration of effective intracellular levels; a depot of zinc dithizonate might retard the rate of loss of dithizone by catabolic mechanisms.

DR. CALVIN: What do you think the zinc cysteinate is doing in the eye?

DR. PHILIPS: Have you ever seen the flash of a cat's eyes at night? This is a reflection from the mirror-like *tapetum lucidum* which lies in the upper part of the eyeground and is composed of a modified layer of choroid cells lying immediately behind the retina. It has been conjectured that the mirror is a device that increases the probability of detection of light of low intensity—a matter of some importance to a nocturnal prowler like the cat. Presumably the retina could be excited by both inwardly bound and outwardly reflected photons. If this is so, it is a remarkable evolutionary adaptation of chelate chemistry.

DR. SEVEN: What is really different about the zinc content of carcinoma cells of the prostate?

DR. THEODORE HALL (Memorial Center, New York): It is true that prostatic cancer tissue has been reported as having a much lower zinc level than normal prostatic material. The data are not good and must be qualified. First, most prostatic cancer patients are old and the zinc level probably drops even in healthy old prostates. Secondly, most of the data in the literature and most of our data are for patients who have been treated by castration, estrogens, or radiation, all of which depress the zinc level. Thirdly, a gross assay of the zinc in the prostatic gland, including connective tissue, does not necessarily reflect the zinc content of the carcinoma cells themselves. Our feeling is that the carcinoma cells probably lose in zinc as they lose differentiation, and the existing data are consistent with this view but far from conclusive.

DR. SEVEN: Why couldn't you use dithizone to transport radioactive zinc to the normal tissue surrounding the carcinomatous tissue?

DR. HALL: Many urologists have been attracted to the possibility of prostatic cancer radiation therapy with radioactive zinc. However, calculations indicate that the radiation dose that could be delivered in this way is therapeutically very marginal, even with the most optimistic assumptions about the amount of radioactive zinc that might be localized favorably. Pilot studies with the rat (Wakeley, J.C.N., B. Moffat, A. Crook and J. R. Mallard. *Intern. J. Appl. Radiation and Isotopes* 7: 225, 1960) suggest an inadequate ratio of dose to the prostate as compared with the dose to nearby healthy tissues and organs.

DR. PHILIPS: I also would like to add that the use of a zinc dithizonate complex for the purpose would be quite impractical. Such complexes are highly insoluble in water and it is doubtful that they would get to prostatic tissue in any significant amount. As for dithizone promoting the entrance of zinc into cells, I have no pertinent information.

DR. SEVEN: There are some compounds which appear to have better permeability when in the form of a metal complex. For example, the lead chelate of EDTA has been reported to go through the gut while EDTA, itself, is not well absorbed. Dr. Calvin, the other day you mentioned modifications of EDTA which might improve its absorption. Could you do anything with dithizone?

DR. CALVIN: It wasn't a modification of the EDTA as a chemical, really, but simply the encasing of it in a microsphere which could be absorbed. We know that polystyrene microspheres can be absorbed. If we encase these chelating agents in a way in which they can be absorbed and dejacket them after absorption, they then could be administered orally.

DR. CHENOWETH: How are you going to get a polystyrenolytic enzyme?

DR. CALVIN: I wouldn't cover it with polystyrene. I would cover it with something that can be dissolved, like fat.

DR. FOREMAN: I would like to add a little bit of trivia to this discussion, too. Actually, lead EDTA does not go across the gut. Studies have been carried out by Clark in one of the drug houses in which she studied the pharmacology of lead EDTA in a similar fashion to our studies of calcium EDTA. She found that only about 2 per cent of the lead EDTA goes across the gut. Secondly, for Dr. Calvin's interest, one of the uses of the *tapetum lucidum* is that it accumulates plutonium vigorously.

DR. SCHUBERT: I would like to ask Dr. Philips what other metals besides zinc are present in these zinc-rich optic tissues? I think of some interesting cooperative effects involving cuprous copper which in combination with zinc, for example, would expedite certain flash phenomena.

DR. PHILIPS: I don't know.

DR. PAUL HAMMOND (University of Minnesota): Recently we attempted experimental treatment of lead poisoning with dithizone in rabbits. We found, much to our surprise, that the dithizone converted a sublethal dose of lead to a lethal one in very rapid and dramatic fashion. I would like to speculate that this is perhaps a case of transfer of lead into cells. I think that there is some other basis for feeling that we can carry heavy metals into cells; for example, there is the work of Gilman several years ago in which he converted a central nervous system type of cadmium toxicity to a nephrotoxicity by the use of BAL. Here he speculated that he was transferring the cadmium into the cells of the kidney tubules.

Design of chelating agents for selected biological activity

ADRIEN ALBERT

Department of Medical Chemistry, Institute of Advanced Studies, Australian National University, Canberra, Australia

THE STRIKING physiological and pathological actions of the cations of metals have created an interest in obtaining metal-binding agents to increase or retard these actions. To be useful, such agents must be selective. It is evident, however, at the present time, that we do not know all the necessary factors to design a specific chelating agent, one whose action will be confined to a single biological result. Fortunately, enough is known to suggest the first steps in such a search, and by making the fullest use of this knowledge the progress of discovery could be greatly speeded. Before we review these known factors, let us review the kinds of selectivity which our advanced technological society is demanding.

Biological selectivity (*requirements*). In agriculture, three classes of selective agents are required. The first class is needed to help the plant select an otherwise inaccessible mineral from the soil. Ethylenediaminetetracetic acid (EDTA) provides an example of this effect. It takes up ferric ions from the soil and transfers them to the plant. If the soil is poor in iron, ferric EDTA is sprayed on the ground with the same good result. A second class of selective agricultural agent is required to differentiate between plants and fungi. For example, copper dimethyldithiocarbamate (DMDC) is widely used to prevent fungal infection of plants. A third class is required to differentiate between plants and insects; but, so far, chelating agents have contributed little to this class.

In veterinary and human medicine, also, three classes exist. The first is required to differentiate between mammals and their parasites. The best known examples are 8-hydroxyquinoline and its halogenated derivatives, and salicylic acid, isoniazid, and the tetracyclines (the latter do not necessarily act entirely by metal-binding). These make a valuable contribution to the fight against fungi and bacteria. No clinically useful chelating agents active against virus, protozoa or worms have yet been discovered although there are promising indications (see below). The second class is required to differentiate between essential metals and poisonous metals. These are the antidotes which include calcium EDTA, which is used to remove lead, and dimercaprol (BAL), which overcomes poisoning by gold, mercury, antimony and arsenic. The third class is required to differentiate between normal and pathological processes, particularly in rheumatic diseases, cardiovascular disease, cancer, and insanity. The use of salicylates in rheumatic diseases and hydralazine in hyperpiesia seem to be the only clinical successes so far recorded in this class, although there are other promising indications (see below). It is only fair to add that the proof that salicylates act by chelation is indirect.

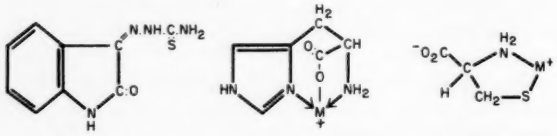
Selectivity achieved by distribution. Selectivity may be claimed when one particular biological site or equilibrium is altered and all others remain unchanged. This desirable state can be accomplished in two different ways, either *a*) through the use of an agent which accumulates only at the site where it is intended to act, or *b*) through the use of an agent which diffuses freely but can respond to biochemical differences between the sites. Method *b*) is dealt with in the next section. Concerning method *a*), it must be pointed out that no differential toxicity need exist in the agent: it can be toxic to all forms of life and yet be highly selective if it accumulates only at the site where interference is required. This principle is illustrated by the antimalarial, pyrimethamine (Daraprim), which is a folic acid antagonist and hence could be highly injurious to all dividing cells in the human body: but it was carefully designed to have a high lipid/water coefficient and other features so that it would accumulate mainly in erythrocytes where the parasites are segregated (33). Pyrimethamine is widely used for the prevention and treatment of malaria and is recognized as a safe and highly specific drug. It does not act by metal-binding, but it provides an excellent example of what can be done by distribution control.

Parasitic bacteria should lend themselves to this approach. They are so small that a large proportion of their vital enzymes are carried on the external cell membrane (which is a lipoprotein mosaic, apparently only four molecules in thickness). Thus, staphylococci have nearly all of their lactic dehydrogenase and succinic dehydrogenase on the external membrane (40). Both of these enzymes should be vulnerable to chelating agents because the former is metal-requiring and the latter has an essential thiol group. The desired selectivity should arise from the fact that, in mammals, these

enzymes are protected by being inside the cell membrane and perhaps may be protected by the mitochondrial membrane as well.

Selectivity achieved through biochemical differences. Comparative biochemistry has revealed some species differences in the use of metals. Thus most molluscs and arthropods use copper in the blood respiratory pigment whereas the majority of animals use iron, mainly as hemoglobin. However, the *Tunicata* or sea-squirts, animals which have backbones only in youth, contain vanadium which seems to take the place of iron in their respiratory pigment (13).

In viruses, the presence of metals seems to be confined to the pox group which contain copper. Although the chemotherapy of virus infections in general has made little headway, dramatic results have been obtained with pox viruses because of their metal-containing peculiarity. Thus mice receiving the chelating agent isatin β-thiosemicarbazone (I) in the diet are protected against ten thousand doses of vaccinia virus (smallpox). These were LD_{50} doses, i.e. each dose was powerful enough to kill one out of every two mice. Yet two doses of 125 mg/kg of this drug saved all the mice, even when the virus was injected intracerebrally. Not only were the mice saved, but they showed no sign of illness at any time (17, 51).



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Some species differences in the metal-binding properties of enzymes have also been recorded. Thus mammalian alcohol oxidase does not contain the free thiol groups found in yeast alcohol oxidase, therefore organic mercurials inhibit the latter but not the former (15). Such qualitative species differences do not seem to be very common (26), but quantitative differences abound. Thus, it is common for an enzyme that is essential for one species to be absent from another. Even tissues of the one species show these quantitative differences. Thus, there is much more glutamine synthetase in (human) brain than in liver, and this enzyme is absent from other tissues. Again, aconitase (an iron-requiring enzyme) is much more abundantly present in heart than in skeletal muscle, whereas the reverse is true for aldolase (believed to be zinc requiring).

The traces of heavy metals in every cell are competed for by a formidable array of natural metal-binding agents. Of these, the amino acids form a useful reference point. Table 1 reports the stability constants for glycine with a number of common metals. The majority of amino acids have figures that differ little from these (2, 3). However, two common amino acids have outstanding binding properties, namely histidine (II) through its imidazole ring, and cysteine (III) through its thiol-group (3). The peptides have lower constants than the amino acids, because the —CO·NH— group does not ionize readily (25, 27). Proteins, in general, have still less avidity, and it appears that the greatest contribution to metal-binding in proteins, such as albumin, is made by cysteine and histidine groups (30, 50). Enzymes, of course, often show a special affinity for a particular metal, e.g. zinc in carbonic anhydrase; or they may work best with a particular metal for which, nevertheless, they do not have the greatest affinity, e.g. magnesium in aldolase. In spite of much speculation, very little is known about the utilization of metals in enzyme action (38). However, vital enzymes seem to bind their metals tightly, at least in microorganisms which powerful chelating agents like 8-hydroxyquinoline can enter without causing harm (18, 28).

Many other cell-constituents bind metals, but porphyrins are unique in their ability to hold iron so strongly that it does not exchange with radioactive iron (31). Purines (4, 12, 32), pteridines (4) and riboflavin (4), also combine with metals. These substances have stability constants of similar magnitude to those of the amino acids, except that ferrous iron is often promoted above its usual position. The other important metal-binding substances in cells are phosphoric acid derivatives and the acids of the citrate cycle. Work has been done on the stability constants of polyphosphates (52) and adenosinetriphosphate (39), but the major part of the phosphate problem, including the nucleic acids, remains unexplored. Similarly, a few constants obtained for the acids of the citrate cycle (46) need supplementing.

Some metal-binding species are confined to certain phyla, the fungi in particular having types (e.g. kojic acid) which are found nowhere else. Chelating agents having special importance in the higher animals include adrenalin (epinephrine), noradrenalin, thyroxine and, perhaps, histamine and spermine.

The selectivity of membranes may provide another important biochemical difference to help in designing selective agents. Thus the complex of ethylenediaminetetraacetic acid (EDTA) with ferric iron does not penetrate bacterial or mammalian cells. But it passes freely through the root hairs of plants and finally appears in the leaves (37).

The literature of comparative biochemistry, if followed closely for new data both on metals and on metal-binding substances in cells, should yield useful clues for increasing biological selectivity.

Chemical selectivity. Biochemists who know of enzymes which are highly specific for a metal are often disappointed to learn that selectivity is quite rare among the man-made reagents for metals. The fact is that most chelating agents show approximately the following order of preference for metals:

Fe^{+3}, Hg^{++}	greatest avidity
Cu^{++}, Al^{+3}	↓
Ni^{++}, Pb^{++}	
Co^{++}, Zn^{++}	
Fe^{++}, Cd^{++}	
Mn^{++}	
Mg^{++}, Ca^{++}	least avidity

So far molybdenum, vanadium, and cuprous copper have not been fitted to this scale.

From our present viewpoint of selectivity, special attention should be given to those very few agents which show a departure from the above order. We might hope, by studying these, to find the cause of the variations and to use this knowledge to find new variants.

Before discussing irregular examples, it would be useful to review the principles of normal chelating behavior. The chemical significance of heavy metals in biology arises from their ability to form bonds that are tighter than ordinary ionic bonds; in other words, they readily form bonds of partly covalent nature. Copper sulfide and the complex cyanides have partly covalent bonds of this kind, but there is greater biological interest in the complexes which metals form with organic substances. When the metal is gripped between any two of the elements: N, O or S, a chelate ring is formed, and the metal is more tightly bound than if it was not part of a ring.

Let us consider the three principal kinds of ligands which bring about chelation. These may have two electron-releasing groups (as in ethylenediamine and 2,2′-dipyridyl) in which case the charge on the metal cation is unaffected by chelation; or there can be one electron-releasing and one anionic group as in glycine, in which case the charge on the metal is diminished by one unit; or there can be two anionic groups as in oxalic acid, in which case the charge on the metal is decreased by two units. In general, chelation through oxygen or nitrogen takes place only when 5- or 6-membered rings can be formed, and of these, 5-membered rings are much more stable. However, chelation through sulfur enables stable 4-membered rings to be formed. The three main types of chelation are illustrated in Figure 1. The arrows in the ring imply that a normally unshared electron-pair of O, N or S is released to the metal.

In the presence of excess ligand, 2:1-complexes can be formed. Ligands of the oxalic acid type use up their charge in forming 1:1-complexes, but these can unite further with the ethylenediamine type, forming a mixed complex (53). A 1:1-complex of the glycine type can combine with another glycine-type ligand. An ethylenediamine type 1:1-complex can combine with any one of the three types of ligands. In general, divalent copper is quadricovalent and is saturated when it has combined with two ligand molecules (whether the same or different). But divalent iron, cobalt and zinc are sexacovalent towards ligands of the ethylenediamine type, and trivalent ions are sexacovalent towards the glycine type as well.

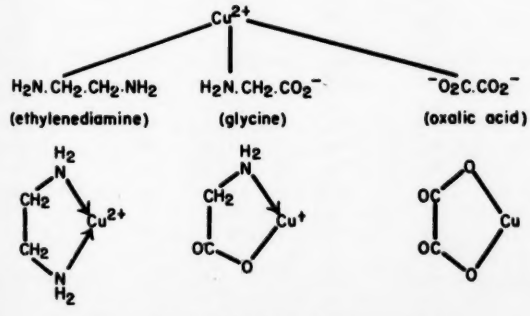


FIG. 1. Three main types of 1:1-complexes.

In order to discuss the various degrees of tightness in binding, and these vary between wide limits, the notion of stability constants was introduced. This is the constant governing the mass-action equilibrium between the ligand(s) and one ion of the metal. Thus for the 1:1-complex of glycine and cupric ion,

$$K_1 = \frac{[H_2N \cdot CH_2 \cdot CO_2Cu^+]}{[Cu^{++}][H_2N \cdot CH_2 \cdot CO_2^-]}$$

and for the 2:1-complex,

$$K_2 = \frac{[H_2N \cdot CH_2 \cdot CO_2CuO_2C \cdot CH_2 \cdot NH_2]}{[H_2N \cdot CH_2 \cdot CO_2Cu^+][H_2N \cdot CH_2 \cdot CO_2^-]}.$$

In each case the product is in the top line, and the substances from which it is formed are in the lower line. For many purposes the overall stability constant (β) is required: this is the product of the individual constants. If there are two individual constants, the product is designated β_2; if there are three constants β_3. Stability constants are usually determined by potentiometric (glass electrode) titration of the ligand in the presence and absence of the metal and combining the results in a set of complex calculations for which simplifying approximations can often be substituted (2–4).

The first stability constant (K_1) is related to two variables ($\bar{n}$) and [L] by the following equation.

$$K_1 = \frac{\bar{n}}{(1 - \bar{n})[L]},$$

where $\bar{n}$ is the average number of complex-forming molecules bound by one atom of metal and [L] is the concentration of the free chelating species of the ligand. Both $\bar{n}$ and [L] depend on the ionization constant of the ligand and on the pH, but each is related to these factors differently. The net result is that an increase in acidity leads to less metal being bound. Figure 2 illustrates a typical formation curve (the plot of $\bar{n}$ against $-\log$ [L]). It will be observed how $\bar{n}$ decreases with falling pH. But the stability constant does not vary with pH, and the use of the above equation will give the same value for K_1 anywhere between $\bar{n} = 0$ and $\bar{n} = 1$, above which a related equation for K_2 is applicable. (If K_1 and K_2 differ by less than a hundredfold, these equations need modification to deal with mutual interference, especially in the $\bar{n} = 0.8$ to 1.2 region.)

The constants of a number of substances of interest to biological workers are given in Table 1.

There is no certain way of predicting the stability constants of a substance in advance of synthesizing it, and making the measurements, all of which is very

time-consuming. The most useful guides in the prediction of approximate stability constants are as follows. In any one series, the more tightly a ligand combines with hydrogen ions (as measured by the pK values) the more tightly will it bind metals. [Present day knowledge of inductive constants makes it easy to forecast ionization constants (as pK_a values) in advance of synthesis.] This rule, which it must be emphasized holds only for a series of related substance, breaks down if bulky groups cause steric hindrance (see below) (35).

When we have to compare members from different series, the most common disturbing factor in the pK_a:log K relationship is the variable resonance in chelated rings. Hence the most useful clue is to examine the double-bond composition of the chelate ring: factors which lower double-bond character usually also decrease the stability (21). Thus, in the copper chelate of acetylacetone (IV) the chelate ring contains two full double bonds and is more tightly bound than in the salicylaldehyde chelate (V) where, on account of the resonance of the benzene ring between the two forms shown in (VI), one of the double bonds is virtually present only half the time. Similar considerations show that in 2-hydroxy-1-naphthaldehyde and 2-hydroxy-3-naphthaldehyde, the shared double bond is present about $\frac{2}{3}$ and $\frac{1}{3}$ of the time, respectively. It was found (21) that the stabilities of the copper derivatives vary accordingly.

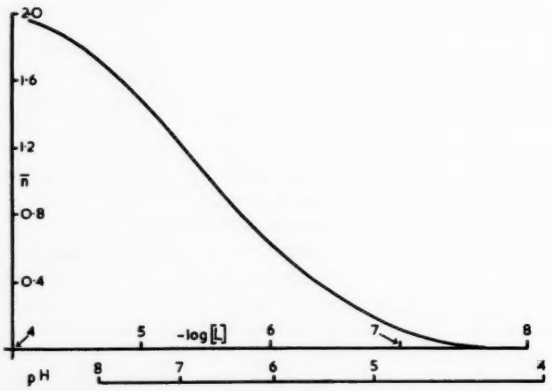
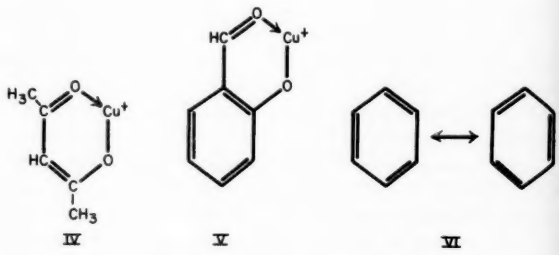


FIG. 2. Formation curve of nickel-glycine. The average number of molecules ($\bar{n}$) of glycine which are united to one atom of metal is plotted against the concentration of the negative logarithm of the chelating species (L) of glycine.



Help in the prediction of stability constants is expected to come from developments of ligand field theory. The core of this treatment is crystal field theory which postulates that the five *d* orbitals of heavy metals, although normally equal in energy, become differentiated when lying in the electrostatic field of the ligand. In particular, those *d* orbitals lying in the direction of the ligands are raised in energy, and those lying away from the ligands are lowered in energy. The donor electrons

TABLE 1. *Stabilility Constants (Logarithmic) of Some Complexing Agents (in Water at 20°)*

Complexes of order 1:1 are reported as log K_1; those of higher order as log β. To save space, the order is given as a pre-index; thus manganese forms only a 2:1-complex with dipyridyl which has log $\beta = 7$: this is reported as $^{2}7$, whereas zinc forms a 3:1-complex of log $\beta = 13$, reported as $^{3}13$.

Ligand	Fe^{+3}	Cu^{++}	Ni^{++}	Zn^{++}	Co^{++}	Fe^{++}	Mn^{++}	Mg^{++}
Glycine	10	8.5; $^{2}15$	6; $^{2}11$	5; $^{2}9$	5; $^{2}9$	4; $^{2}8$	3; $^{2}5.5$	2; $^{2}4$
Cysteine	*	*	10; $^{2}19$	10; $^{2}18$	$^{3}16$	6	4	<4
Histidine	?	10.5; $^{2}19$	9; $^{2}16$	7; $^{2}12$	7; $^{2}13$	5; $^{2}9$	<4	<4
Histamine	?	10; $^{2}16$	7; $^{2}11$	5; $^{2}9$	5; $^{2}9$	4	?	?
Ethylenediamine	?	11; $^{2}20$	8; $^{3}18$	6; $^{3}12$	6; $^{3}14$	4; $^{3}9.5$	3; $^{2}5$	?
Ethylenediaminetetraacetic acid (EDTA)	24	19	18	16	16	14	13.5	9
Pteroylglutamic acid ("folic acid")	?	$^{2}8$	$^{2}9$	$^{2}7.5$	$^{2}8$	$^{2}8$	$^{2}6$	?
Hypoxanthine	?	6	5	?	4	4	2	?
Guanosine	?	6	4	4.5	3	4	3	?
Adenine	?	$^{2}14.2$	4.4	?	4.2; $^{2}8.4$	?	?	?
Riboflavin	?	$^{2}13$	4	5.5	4	7	3	?
8-Hydroxyquinoline (oxine)	12; $^{2}24$; $^{3}36$	12; $^{2}23$	10; $^{2}18$	8.5	9; $^{2}17$	8; $^{2}15$	7; $^{2}12$	4.5
o-Phenanthroline	$^{3}14$	$^{3}20$	$^{3}24$	$^{3}17$	$^{3}20$	$^{3}21$	$^{2}8$	?
Dipyridyl	?	$^{3}18$	?	$^{3}13$	?	$^{3}17$	$^{2}7$	?
Oxalic acid	10	6	5.5	5	4.5	4.5	4	(Ca. = 3)
Salicylic acid	16; $^{2}28$	11; $^{2}19$	7; $^{2}12$	7	7; $^{2}11$	6; $^{2}11$	6; $^{2}10$	?
Tetracycline	10; $^{3}25$	8; $^{2}13$	6; $^{2}11$	5; $^{2}9$	5; $^{2}10$	5; $^{2}9$	4; $^{2}8$	4
Isonicotinic hydrazide	?	8	5.5	5	5	?	?	?

* Cysteine is oxidized by this cation.

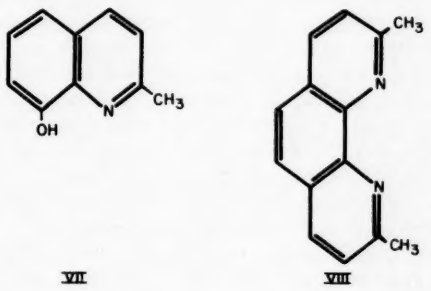
on the ligands repel the *d* electrons of the metal: this repulsion is reduced by the movement of *d* electrons into those *d* orbitals which are further from the ligands. The energy liberated by this effect is called the crystal field stabilization energy. It varies with the ligand and the number of *d* electrons, and it can be calculated in simple cases. Although evolved for crystals, crystal field theory is applicable to complexes in solution. For aromatic ligands and certain metals (notably iron, nickel and cobalt) allowance must be made for π-bonding. With this modification, crystal field theory has been combined with molecular orbital theory to give what is called ligand field theory, which shows promise of being a great practical help to the student of chelation phenomena. For further reading, see reference (16).

Having reviewed the regular features of affinity, we are in a position to assess some of the irregular features. Copper forms planar complexes, iron (both Fe^{++} and Fe^{+3}) octahedral complexes, and the other metals usually tetrahedral complexes. This means that copper is uniquely sensitive to steric effects. Thus, in both folic acid and *o*-phenanthroline, the stability constants for copper lie below those for nickel (see Table 1) which is contrary to the natural order discussed above.

Another type of steric effect arises when a bulky group is inserted near to the chelating groups. This prevents two ligands from approaching close enough to form a strong 2:1-complex, and, in the case of metallic cations of small diameter, the cation cannot be gripped. Thus, 2-methyloxine (VII) binds all cations less strongly than oxine does, but the difference is greatest for aluminum (34, 35) which has the smallest crystal radius (0.57A) of any common metal (see Table 2). Again, two molecules of 2,9-dimethyl-*o*-phenanthroline (VIII) hinder one another too much to retain ferrous ions (radius 0.83A), but the larger cuprous ion (radius 0.96A) is held well (56). Similarly, magnesium is usually more tightly bound than calcium, but its radius is 0.28A smaller than that of calcium so that steric hindrance in the approach of two molecules of a bulky ligand provides a system that binds calcium preferentially (55). Some relevant radii are given in Table 2. It should be noted that Fe^{++}, Co^{++}, Ni^{++} and Zn^{++} have almost the same diameters and could not be separated through steric hindrance.

TABLE 2. *Crystal Radii of Cations (in A)*

Li^{+}	0.78	Be^{++}	0.31	Al^{+3}	0.57
Na^{+}	0.98	Mg^{++}	0.78	Fe^{+3}	0.67
K^{+}	1.33	Ca^{++}	1.06	Ga^{+3}	0.62
Rb^{+}	1.49	Sr^{++}	1.27	Tl^{+3}	1.05
Cs^{+}	1.65	Ba^{++}	1.43		
Cu^{+}	0.96	Mn^{++}	0.91		
Tl^{+}	1.49	Fe^{++}	0.83		
Ag^{+}	1.13	Co^{++}	0.82		
		Ni^{++}	0.78		
		Cu^{++}	~0.72		
		Zn^{++}	0.83		
		Cd^{++}	0.72		
		Hg^{++}	1.12		
		Pb^{++}	1.32	Zr^{+4}	0.80



VII VIII

A selective enhancement of the bonding of iron is found in aromatic ligands when the geometry is favorable. This is caused by "back double-bonding," i.e. a π-bonding in which unused electrons in a suitably placed 3 *d* orbital are contributed by the iron to empty molecular orbitals in the ligand. This reinforces the ligand-metal bonds and hence greatly increases the stability of the complex. Ferrous iron usually gives complexes which are paramagnetic and almost colorless. But with aromatic ligands, particularly those containing nitrogen, deeply colored complexes are formed with loss of paramagnetism. However, the effect is seen in a higher degree in 4-hydroxypteridine, riboflavin, folic acid and *o*-phenanthroline where the constants for ferrous iron greatly exceed those for zinc. Nickel and cobalt are also capable of back double-bonding, but to a smaller extent.

Another kind of differentiation is seen in the plot of stability constants against atomic number in the series: manganese, iron, cobalt, nickel, copper and zinc (see Fig. 3). This curve rises most steeply for ligands that

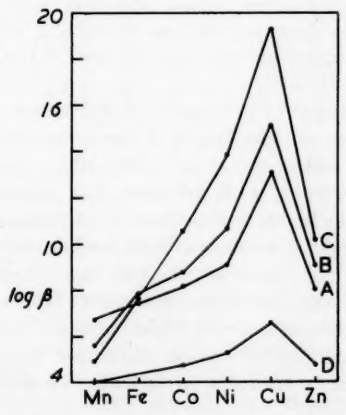


FIG. 3. The relative affinities of cations for oxygen and nitrogen types of chelating agent. The logarithms of the stability constants are plotted against rising atomic numbers. *A*: Salicylaldehyde; *B*: glycine; *C*: ethylenediamine; *D*: oxalic acid.

chelate by two nitrogen atoms (e.g. ethylenediamine), less steeply for those that chelate with one nitrogen and one oxygen atom (e.g. salicylaldehyde, or glycine) and least steeply for those which chelate by two oxygen atoms (e.g. oxalic acid). The curves for substances of these different classes often cross one another, as happens in Figure 3 with ethylenediamine and salicylaldehyde. Thus, in a solution of these two substances, all manganese would be bound by the salicylaldehyde, iron would be divided between both, and the heavier metals would be held mainly by the ethylenediamine. Not only Mn^{++}, but Mg^{++}, Ca^{++}, Fe^{+3}, Tl^{+3}, Mo^{+5} and Va^{+5} show this preference for oxygen over nitrogen.[1]

Most metals prefer combination with oxygen to sulfur, but Cu^{+}, Ag^{+}, Au^{+}, Hg^{++}, As^{+3} and Sb^{+3} prefer sulfur to oxygen thus explaining the successful use of dimercaprol as an antidote for poisoning by some of these cations. Three other cations, Cu^{++}, Ni^{++} and Co^{++} slightly prefer sulfur to oxygen especially if the sulfur is nonionic, as in an organic sulfide.

Another factor, which can cause the relative affinities of a series of metals to change, is that the oxidation-reduction potential of a metal is always altered by chelation. Although such an alteration can occur only in a metal of variable valence (e.g. Cu, Fe, Co, Mn, Mo, V), it can have the result that the metal quickly changes, after chelation, to a valence higher or lower than the one originally chosen. In its new valence, the metal is likely to belong to a quite different part of the affinity scale.

Although the study of this effect is only in its infancy, something of the scope can be gleaned from Table 3 (it should be borne in mind that the scale of potentials ranges from about +2 volts for the most powerful oxidizing agents to about −2 volts for the most powerful reducing agents). Cobalt provides an interesting example of this effect. Normally cobaltous salts are stable in aqueous solution whereas a cobaltic salt is instantly decomposed by water with the evolution of oxygen. However, upon chelation with ethylenediamine the potential falls so drastically that the cobaltous complex becomes readily oxidized to the more stable cobaltic state.

The potential of a complex is determined by *a*) the ionic charge on the ligand, *b*) back-bonding of metal to ligand, and *c*) the crystal field effect (42). For example, concerning *a*) it is known that when the ligand is anionic the higher valence state of the metal is favored; concerning *b*) it is known that back-bonding to sulfur favors the lower valences; and concerning *c*) it is known that the crystal field effect favors Cu^{++} and Fe^{++} to other valences of these metals. Unfortunately, these factors cannot yet be integrated to predict the potential of a complex in advance of measurement.

[1] The removal of a pathological excess of iron from the human body has been attempted with EDTA and related substances. It may be useful to try substances with a steeper curve, e.g. sulfonic acids derived from oxine and *o*-phenanthroline.

TABLE 3. *Effect of Ligands on Oxidation-Reduction Potentials* (42)

Simple Ion	E_0 (Potential in Volts)	Complexes* With:	E_0
$Fe(H_2O)_6^{++} \rightleftharpoons Fe(H_2O)_6^{+3}$	+0.77	[1]EDTA	+0.14
		[1]Salicylic acid	+0.20
		[6]Cyanide ion	+0.36
		[1]Glycine	+0.38
		[1]Oxine	+0.52
		[1]Oxytetracycline	+0.57
		[3]Dipyridyl	+1.06
		[3]*o*-Phenanthroline	+1.06
$Co(H_2O)_6^{++} \rightleftharpoons Co(H_2O)_6^{+3}$	+1.84	[6]Cyanide ion	−0.83
		[3]Ethylenediamine	−0.22
		[6]Ammonia	+0.14
$Cu(H_2O)_x^{+} \rightleftharpoons Cu(H_2O)_4^{++}$	+0.17	[2]Ethylenediamine	−0.38
		[2]Glycine†	−0.16
		[2]Dipyridyl†	+0.12
		[2]Ammonia	+0.34

* The pre-indexes have the same meaning as in Table 1. † I am grateful to Dr. R. J. P. Williams for allowing me to present these figures in advance of publication.

A number of oxidation-reduction potentials are given in Table 3.

It is evident from Table 3 that some complexes are more powerful oxidizing agents than the free metal cations. Such complexes could cause the destruction of vital metabolites. Thus, the oxine ferric complex catalyzes the aerial oxidation of the thiol-groups in liver nucleoprotein, whereas inorganic Fe^{+3} is ineffective (20). It has been suggested that oxine acts similarly when functioning as an antibacterial or fungicide (7). The diethyldithiocarbamates are believed to destroy thioctic acid (47). In both cases, the chelating agent acts only after combination with a metal of variable valence (Fe, Cu). For further discussion of this effect, see below under "Mode of action." It has also been suggested that when isoniazid is oxidized inside the tubercle organism the reaction is metal catalyzed and a destructive free radical is formed (57). The increase in the chemical reactivity of metals after chelation is not entirely confined to polyvalent metals. Chelation imposes an electronic redistribution on the metal, and sometimes a change in geometry, both of which can increase chemical reactivity in suitable cases.

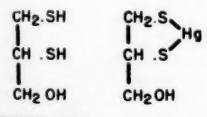
This chemical section concludes with a caution that stability constants are not always the best measure of the relative competing value of two chelating agents (say, a drug and a metabolite). At a given pH, these agents are likely to be ionized to different extents. The one which has the less affinity for metals (as shown by the lower stability constant) may, through a difference in pK_a, produce, for instance, a hundred times as much anion as the other agent. In this case, the substance with the less affinity for the metal may well be the one that gets most of it. This is so because chelation requires not only an affinity between agent and metal, but also a ready supply of anions from the agent (or of

neutral molecules, if the agent is a base). Fortunately, this possible competition between stability constant and ionizing constant does not occur within a series of chemically closely related ligands, as was explained above.

Modes of biological action. Two entirely different modes of biological action are recognized among chelating agents. Some act by immobilizing a metal, but many others exert their biological activity only in conjunction with a metal, i.e. by a cooperative effect.

The simplest kind of action is shown by the antidotes, which are used in occupational poisoning, also in cases of overdosage of drugs, and also to hasten the excretion of radioactive elements. Thus dimercaprol (IX) binds gold, mercury, antimony and arsenic in the blood stream. It forms complexes, e.g. (X), the main feature of which is that a bondable hydrogen atom remains free to assist excretion by the kidneys. The use of penicillamine, in Wilson's disease, to remove excess of copper from the body is similar, also the calcium chelate of EDTA (XI) in lead and vanadium poisoning. Large doses of sodium salicylate have proved effective in beryllium poisoning.

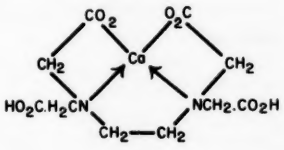
The successful use of 2-mercaptoethylamine (cysteamine) to prevent irradiation injury may rest on its ability to break the destructive chain reaction of hydroxyl radicals which is apparently propagated by traces of iron or copper. S:2-Aminoethyl*iso*thiuronium salts, used for the same purpose, generate 2-mercaptoethyl-guanidine which should act similarly.



DIMERCAPROL

IX

X



XI

Experience with the clinical use of antidotes shows that chelating agents can circulate in the blood stream without depleting the body of essential heavy metals. This is, of course, a matter of carefully controlled dosage. The provision of bondable hydrogen atoms (i.e. hydrogen on O, N or S) ensures that little of the antidote enters cells, and that it is rapidly excreted. In the absence of heavy metals, a chelating agent may not necessarily damage those cells into which it penetrates. Thus oxine (18, 28) was found to do no harm inside bacterial (18) and fungal (28) cells, but if inorganic iron or copper (respectively) were made available at the same time, the organism was quickly destroyed.

When it was found in 1944 (1) that 8-hydroxyquinoline (oxine) acts by chelation, a prospect was opened up that other antibacterial and antifungal drugs might be found to act similarly. Many antiparasitic chelating agents are now known and the well investigated ones have been shown to act in a different way from the antidotes. They do not remove an essential metal, but form a lethal complex with a metal accidentally present. Only metals of variable valence, chiefly iron and copper, activate these agents. Oxine and DMDC, which are typical examples, have tremendous economic importance and both are manufactured in vast quantities. Oxine is used for rot-proofing structural materials, e.g. canvas for tents, and nowadays copper is always added. Oxine and its 5,7-dihalogenated derivatives ("Vioform," "Quinolor") are much used in human medicine, particularly for bacterial and fungal infections of the skin. The various salts of dimethyldithiocarbamic acid, some specially chosen for their adhesive properties, are used on a vast scale for protecting crops against fungi.

It is known that cell membranes carefully regulate the intake of heavy metal cations, and even cations essential in traces for nutrition are toxic in excess. But uncharged complexes are highly liposoluble and hence should readily penetrate cell-membranes in a way which the cell may not be able to regulate. Reference to Figure 1 shows that, for a divalent metal, all complexes of the oxalic acid type, and 2:1 complexes of the glycine type, are uncharged and hence should penetrate cells readily. (It should be noted that polydentate substances like EDTA (XI) often have groups in excess of those with which a metal can combine, and the complexes would not be expected to penetrate ordinary cell membranes.) These highly liposoluble complexes enable the metal to transport the chelating agent (or, conversely, the chelating agent to transport the metal) into the cells in quantities larger than would normally occur.

It seems that the chelated complex is usually the active toxic agent. Thus oxine (XII), 2-mercaptopyridine-N-oxide (XIII) and DMDC (XIV) are not toxic on their own but become highly toxic when combined, whether accidentally or intentionally, with iron or copper. The mode of action of oxine has recently been reviewed in detail (7), and hence only the major features will be described here.

Table 4 demonstrates the innocuousness of oxine and iron separately, and their toxic nature when combined; the organism being *Staphylococcus aureus* in distilled water (8). When broth was used instead of water, the iron (naturally present) might give the misleading impression that oxine is toxic on its own. Copper is the only other cotoxicant metal for bacteria, and the only one for fungi when oxine is used. It will be seen from Table 4 that 2-mercaptopyridine-N-oxide exhibits antibacterial action only in the presence of a metal (iron) (11). Table 5 reveals the necessity of a metal (copper) for the action of DMDC on *Aspergillus niger*. Several other moulds behave similarly (47). A feature of Table 5 is that the effective proportions of metal and chelating agent are critical. This has also been demonstrated for oxine (8) and 2-mercaptopyridine-N-oxide (11), and the interpretation seems to be that the 1:1-complexes are by far the more toxic, but they enter the cells only as the 2:1 (agent: metal) complexes. In the case of

TABLE 4. *The Innocuousness of Some Chelating Agents in the Absence of Iron (8, 11) (Bactericidal Test)*

Staph. aureus: pH 6–7 (20°)

Oxine 1/M*	Fe^{++}, or Fe^{+3} 1/M*	Growth (Plated Out After 1 Hour)	
		Glass Distilled Water	Meat Broth
nil	nil	+++	+++
100,000	nil	+++	−
nil	100,000	+++	+++
100,000	100,000	−	−
2-Mercaptopyridine-N-oxide	Fe^{+3}	Glass Distilled Water	
80,000	nil	+++	
nil	80,000	+++	
80,000	80,000	−	

+++ means prolific growth with uncountable colonies. −means no detectable growth. * Dilution expressed as 1/molarity.

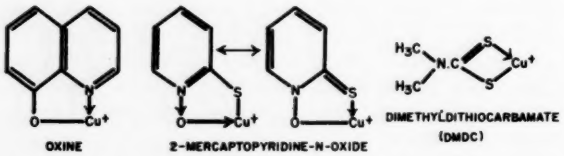
TABLE 5. *Effect of Copper on Growth Inhibition in Aspergillus niger by Sodium Dimethyldithiocarbamate (47)*

(3 days at 24°)

Glucose, salts, and vitamins in glass-distilled water at pH 7

Cu^{++} 1/M	Sodium DMDC 1/M							
	nil	700,000	280,000	140,000	70,000	28,000	14,000	7,000
nil	+	+	+	+	+	+	+	+
83,000	+	+	+	+	+	+	+	+
25,000	+	+	−	−	−	±	+	+

DMDC, the 2:1 copper complex has an extraordinarily low solubility in water (0.01 part per million) which restricts the supply to the cell. The diethyl homologue is soluble only 0.002 p.p.m. and is almost inactive (47).



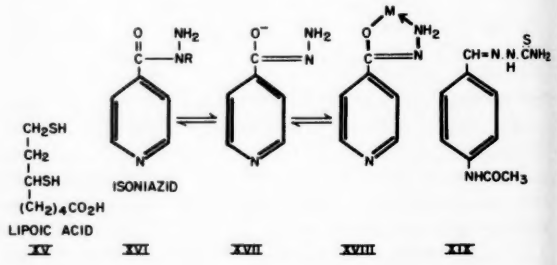
Note that (XII), (XIII) and (XIV) are shown as their 1:1-complexes. A typical equilibrium (instantaneous) between the 1:1- and 2:1-complexes is exemplified below.

$$(\text{oxine})Cu^{+} \underset{+Cu^{++}}{\overset{+\text{oxine anion}}{\rightleftharpoons}} (\text{oxine})_2Cu$$

1:1-complex 2:1-complex

It has been suggested (47) that (XII), (XIII) and (XIV) all act by catalyzing the oxidative destruction of lipoic acid (thioctic acid; XV), which is the essential coenzyme for the oxidative decarboxylation of pyruvic acid (29). There is no doubt that they often cause pyruvic acid to accumulate, but it seems surprising that this could cause the death of bacteria within 10 minutes, which oxine does.

That a metal can become much more chemically active after chelation is evident enough in the oxygen-binding properties of hemoglobin and the oxidizing powers of the heme enzymes. Many examples of this effect are known among quite simple chelating agents (7). Thus, it is quite reasonable to suppose that the destructive action brought about by oxine is due to the intact oxine-metal complex. However, the possibility exists that the oxine is metabolized inside the cell, and that inorganic iron (imported in excess) is the true toxicant. This possibility has been discussed for 2-mercaptopyridine-N-oxide because it is easily destroyed by oxidation (11). It has a good precedent in the use of ferric EDTA to transport ferric ions into plants: in the leaves the EDTA-iron complex is destroyed and inorganic iron liberated (37). Other chelating substances exist whose mode of biological action is known incompletely. Salicylic acid and its derivatives long have been used as fungicides and in the treatment of rheumatic diseases. The chelating powers of salicylic acid, which are high (41), depend on the ionization of the phenolic group. The esters and the corresponding aldehyde have less avidity for metals, and the amides have none at all (41). Thus, the antifungal action of salicylanilide, so much used to prevent mildew on textiles, probably arises after partial metabolism by the fungi.



Tetracycline and its derivatives, such as "Aureomycin," have about the same avidity as glycine for divalent metals and form unusually stable complexes with trivalent metals (10). The mode of action of these chelating agents is unknown, but it must be different from that of oxine because it is much slower and takes place in the absence of iron. The action of tetracyclines on bacteria and fungi is often specifically inhibited by ferrous ions. (For stability constants of a typical tetracycline, see Table 1.)

The evidence that streptomycin may act through chelation has been reviewed (54).

Isoniazid, or isonicotinic hydrazide (XVI, R = H), which is the most important drug for treating human

tuberculosis, has an affinity for metals of about the same order as glycine (see Table 1) (5). In order to form complexes (XVIII), isoniazid must first form the anion (XVII). However no anion can be formed from 1-isonicotinyl-1-methylhydrazine (XVI, R = CH_3). The inactivity of this substance *in vitro* is good evidence that isoniazid acts in tuberculosis through chelation (23, 24).

However, its action cannot be due to chelation alone, because when the side chain is moved from the 4- to the 2-, or 3-position of the pyridine nucleus, the isomers so produced have little action against *M. tuberculosis*, even *in vitro*, although their stability constants are no lower (6). It is known that isoniazid enters the cells of nonresistant tubercle bacteria, and does not penetrate the cells of resistant strains; also bacterial species such as *Staph. aureus* and *E. coli*, which are never affected by isoniazid, do not absorb it (58). It seems clear that the lethal action on the tubercle bacillus takes place inside the cell. The possibility that death of the bacillus follows the metal-induced formation of free-radicals was mentioned above (57). It would be interesting to know if isoniazid is excluded from the human cell: if so, an intensive study of what causes it to penetrate susceptible bacterial cells would be rewarding; if not, attention would be focused on metabolic differences between human cells and those of the tubercle bacillus.

p-Acetamidobenzaldehyde thiosemicarbazone ("Tibione"; XIX) is active against strains of *M. tuberculosis* that are fully resistant to isoniazid. Hence the two drugs cannot act by the same mechanism.

A novel use of a chelating agent in medicine is provided by tetraethylthiuram disulfide (disulfiram, antabuse) which is given to persuade alcoholics not to drink fermented beverages. It inhibits an aldehyde dehydrogenase in the liver, and hence the acetaldehyde content of the blood reaches 5 to 10 times the normal value which the patient finds highly unpleasant. This substance, first used as a fungicide in agriculture, becomes reduced to DMDC before acting. The enzyme inhibited in the liver seems not to be a metal-requiring one, but it has essential thiol-groups: these may be oxidized by a DMDC-metal complex.

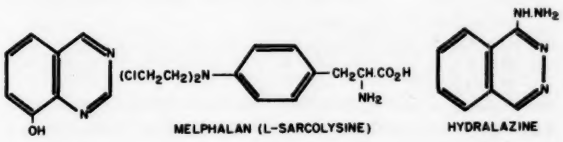
Some fundamentals in designing new chelating agents. It is doubtful if a metal-binding agent could be successful, in a biological environment, unless its stability constants were at least as high as those of the common amino acids. However, it does not follow that only agents with very high constants should be considered, because ease of cation exchange is essential in some applications (45).

Actually, few substances having very high stability constants are known. Many of these are polydentate ligands, and these are undesirable when it is intended to leave some of the valences of the metal exposed for combining with a metabolite. In this connection, it is noteworthy that *mixed* complexes are easily formed, e.g. between a metal and one molecule each of oxalic acid and ethylenediamine (53). Enzyme-metal-substrate complexes may be of this type.

TABLE 6. *Fall and Rise of Bacteriostatic Action With Fall and Rise of Partition Coefficient* (9)
Strept. pyogenes in meat broth at pH 7.3 (37°)

Substance	Partition Coefficient Oleyl Alcohol/Water	Lowest Inhibitory Dilution 1/M	log First Stability Constant (Ni^{++})
Oxine	67	200,000	9.8
2-Aza-oxine	6	13,000	7.8
3-Aza-oxine	5	13,000	7.6
4-Methyl-2-aza-oxine	16	25,000	8.1
4-Methyl-3-aza-oxine	17	50,000	7.9
4-Propyl-3-aza-oxine	135	100,000	7.9

At some stage in the design, it must be decided whether penetration into cells is to be desired or to be avoided. If desirable, lipophilic groups should be added to the molecule. For this purpose carbon, halogens, hydrogen and sulfur are considered as lipophilic and nitrogen and oxygen as hydrophilic; the effect can be followed by a study of oil/water partition coefficients. Complexes of the ethylenediamine type (see Fig. 1), which preserve the original charge on the metal, would need to be supplied with many more lipophilic atoms to acquire ease of penetration than would be necessary for an uncharged complex. Apparently dipyridyl and *o*-phenanthroline (both of which are of the ethylenediamine type) have the required balance for at least one type of membrane: they can penetrate the cuticle of worms and paralyze the neuromuscular apparatus (14). Oxine readily penetrates cells. The effect of adding hydrophilic and then lipophilic groups to oxine is illustrated in Table 6.



XX XXI XXII

It is evident from Table 6 that very small changes in a molecule can produce large changes in partition coefficients. Thus, the insertion of an extra ring-nitrogen into oxine to give 3-aza-oxine (8-hydroxyquinazoline, XX), makes the molecule much more hydrophilic and lowers the oil-water partition coefficient from 67 to 5. Keeping step with this change, the antibacterial action decreases to one-sixteenth (9). The addition of a side chain of only three carbon atoms (the propyl group) restores the partition coefficient, and the antibacterial titer rises to a value only one tube short of oxine. (That it does not quite reach the value of oxine is due to the stability constants being permanently lowered by the extra ring-nitrogen, a cation-repelling group.)

Uptake by cells might be sought in another way,

by using ligands which resemble natural substrates such as amino acids. Apparently the penetration of drugs of the nitrogen-mustard class has been increased in this way, e.g. the phenylalanine derivative, melphalan (XXI), also known as "Alkeran," or (in the racemic form) sarcolysine (19).

Many excellent chelating agents are unsuited for internal therapy in man simply because they are not selective enough. One must proceed cautiously with those metal-binding substances, including oxide and DMDC, which injure the islets of Langerhans and thus cause diabetes in experimental animals (36).

Some promising medical applications. Many medical applications of chelating agents are likely to be derived from modification of agents already in use (e.g. tetracycline). However, there are many clues for development in quite new fields as the following examples indicate.

Mice have been protected against two typical malignancies (Ehrlich carcinoma and Crocker sarcoma 180) by injection of various copper chelates, particularly copper dimethylglyoxime in 0.002% solution (48, 49).

Many chelating agents have been shown to reduce blood pressure without acting through the nervous system. These include hydralazine (XXII), 2,3-dimercaptopropanol (IX), sodium azide, thiocyanate and nitroprusside (in man), and hydralazine, oxine-5-sulfonic acid and ethylenediaminetetracetic acid (in mice) (44). The mode of action is, presumably, blockage of the enzymes which decarboxylate amino acids to pressor amines. Thus, hydralazine (10^{-4} M) was found to halve the decarboxylation rate of dihydroxyphenylalanine by guinea pig kidney, but this reaction was not inhibited by EDTA, oxine-5-sulfonic acid, or sodium azide. The oxidation of tryptamine by kidney, an example of the destruction of a pressor amine in nature, was found to be greatly and specifically accelerated by vanadium (43).

Cardiovascular degeneration in man is accompanied by increased synthesis of cholesterol from acetic acid. This synthesis has been shown to be catalyzed (in rats) by manganese and retarded by vanadium or iron. Oxine, given as 0.1 per cent of the diet for 10 days, decreased the incorporation of acetate into cholesterol, apparently by chelating manganese (22).

Other interesting applications include the treatment of cardiac diseases with EDTA, the investigation of the level of copper protein in various disease states, and the attempt to link dental caries with chelation. Workers concerned with the application of chelating agents can look forward to a long period of increasing expansion.

REFERENCES

1. Albert, A. *M. J. Australia* 1: 245, 1944.
2. Albert, A. *Biochem. J.* 47: 531, 1950.
3. Albert, A. *Biochem. J.* 50: 690, 1952.
4. Albert, A. *Biochem. J.* 54: 646, 1953.
5. Albert, A. *Experientia* 9: 370, 1953.
6. Albert, A. *Nature, London* 177: 525, 1956.
7. Albert, A. *Selective Toxicity* (2nd ed.). London: Methuen, 1960.
8. Albert, A., M. Gibson and S. Rubbo. *Brit. J. Exper. Path.* 34: 120, 1953.
9. Albert, A., A. Hampton, F. Selbie and R. Simon. *Brit. J. Exper. Path.* 35: 75, 1954.
10. Albert, A. and C. Rees. *Nature, London* 177: 433, 1956.
11. Albert, A., C. Rees and A. Tomlinson. *Brit. J. Exper. Path.* 37: 500, 1956.
12. Albert, A. and E. Serjeant. *Biochem. J.* 76: 621, 1960.
13. Baldwin, E. *An Introduction to Comparative Biochemistry.* London: Cambridge Univ. Press, 1948.
14. Baldwin, E. *Brit. J. Pharmacol.* 3: 91, 1948.
15. Barron, E. S. G. *Advances Enzymol.* 3: 149, 1943.
16. Basolo, F. and R. Pearson. *Mechanisms of Inorganic Reactions.* New York: Wiley, 1958.
17. Bauer, D. and P. Sadler. *Brit. J. Pharmacol.* 15: 101, 1960.
18. Beckett, A., A. Vahora and A. Robinson. *J. Pharm. & Pharmacol.* 10: 160T, 1958.
19. Bergel, F. *Ann. New York Acad. Sc.* 68: 1244, 1958.
20. Bernheim, M. and F. Bernheim. *Cold Spring Harbor Symposia Quant. Biol.* 7: 174, 1939.
21. Calvin, M. and K. Wilson. *J. Am. Chem. Soc.* 67: 2003, 1945.
22. Curran, G. L. *Proc. Soc. Exper. Biol. & Med.* 88: 101, 1955.
23. Cymerman-Craig, J. and D. Willis. *J. Chem. Soc.*, p. 4315, 1955.
24. Cymerman-Craig, J., S. Rubbo, D. Willis and J. Edgar. *Nature, London* 176: 35, 1955.
25. Datta, S. P. and B. Rabin. *Biochim. et biophys. acta* 19: 572, 1956.
26. Dixon, M. and E. C. Webb. *Enzymes.* London: Longmans, 1958.
27. Dobbie, H. and W. Kermack. *Biochem. J.* 59: 246, 1955.
28. Greathouse, G., S. Block, E. Kovach, D. Barnes, C. Byron, G. Long, D. Gerber and J. McLenny. *Research on Chemical Compounds for Inhibition of Fungi*, U. S. Corps of Engineers, Fort Belvoir, Virginia, 1954.
29. Grisebach, H. *Angew. Chem.* 68: 554, 1956.
30. Gurd, F. and D. Goodman. *J. Am. Chem. Soc.* 74: 670, 1952.
31. Hahn, P., W. Bale, J. Ross, R. Hettig and G. Whipple. *Science* 92: 131, 1940.
32. Harkins, T. and H. Freiser. *J. Am. Chem. Soc.* 80: 1132, 1958.
33. Hitchings, G. *Tr. Roy. Soc. Trop. Med. & Hyg.* 46: 467, 1952.
34. Irving, H., E. Butler and M. Ring. *J. Chem. Soc.* p. 1489, 1949.
35. Irving, H. and H. Rossotti. *Acta Chem. Scand.* 10: 72, 1956.
36. Kadota, I. and T. Abe. *J. Lab. & Clin. Med.* 43: 375, 1954.
37. Krugers, J. and J. Agterdenbos. *Nature, London* 179: 45, 1957.
38. Malmström, B. and A. Rosenberg. *Advances in Enzymol.* 21: 131, 1959.
39. Martell, A. and G. Schwarzenbach. *Helv. Chim. Acta* 39: 653, 1956.
40. Mitchell, P. and J. Moyle. *Biochem. J.* 64: 19P, 1956.
41. Perrin, D. D. *Nature, London* 182: 741, 1958.
42. Perrin, D. D. *Revs. Pure and Appl. Chem.* (Australia) 9: 257, 1959.
43. Perry, H., S. Teitlebaum and P. Schwartz. *Fed. Proc.* 14: 113, 1955.
44. Schroeder, H., H. Perry and E. Menhard. *J. Lab. & Clin. Med.* 46: 416, 1955.
45. Schubert, J. *Chimia* 11: 113, 1957.
46. Schubert, J. and A. Lindenbaum. *J. Am. Chem. Soc.* 74: 3529, 1952.
47. Sijpesteijn, A. K. and M. Janssen. *Antonie van Leeuwenhoek* 25: 422, 1959.
48. Takamiya, K. *Gann* 50: 277, 291, 1959.
49. Takamiya, K. *Nature, London* 185: 190, 1960.
50. Tanford, C. *J. Am. Chem. Soc.* 74: 211, 1952.

51. THOMPSON, R. L., S. MINTON, J. OFFICER AND G. HITCHINGS. *J. Immunol.* 70: 229, 1953.
52. VAN WASER, J. AND D. CAMPANELLA. *J. Am. Chem. Soc.* 72: 655, 1950.
53. WATTERS, J. AND R. DE WITT. *J. Am. Chem. Soc.* 82: 1333, 1960.
54. WEINBERG, E. *Bact. Rev.* 21: 46, 1957.
55. WILLIAMS, R. J. P. *J. Chem. Soc.* p. 3770, 1952.
56. WILLINK, H. AND J. WIBAUT. *Rec. trav. chim.* 54: 275, 1935.
57. WINDER, F. AND J. DENNENY. *Biochem. J.* 73: 500, 1959.
58. YOUATT, J. *Australian J. Exper. Biol. & M. Sc.* 36: 223, 1958.

DISCUSSION

Design of chelating agents for selected biological activity

WILLIAM O. FOYE
Massachusetts College of Pharmacy, Boston, Massachusetts

SEVERAL FACTORS can be profitably taken into consideration in the design of chelating agents for specific biological effects. This discussion will be limited in this regard to the use of drugs in the animal organism. Those factors that deserve some attention include the following: factors that affect the strength of metal-binding, factors that direct the metal-ligand ratio, and factors that influence the nature of the solubility of either the chelate or chelating agent. Other factors of equal importance no doubt exist, but those mentioned have been employed the most frequently in the modification of chelating agents. Resolution of these effects in terms of the underlying factors has not been too successful, nor have the explanations relating chelation mechanisms to drug activity been completely convincing. However, keeping the above factors in mind has been and probably will continue to be helpful in the biological study of chelators.

The factors which control the extent of metal-binding by a chelating agent may be either electronic or steric in nature, and have been adequately described. Numerous studies have been undertaken to ascertain the effect of variously placed substituents on the chelating ability of a given molecule. This has been done in the case of a biologically active compound with salicylic acid by including both electron-attracting and electron-repelling groups in the 3, 4, and 5 positions (18).

It had been found previously (15, 16) that salicylic acid chelates of appreciable stability (greater than that of common amino acid chelates) were formed only with ferric, aluminum, and cupric ions. Stability constants were obtained for the chelates of the substituted salicylic acids and these ions. Large differences in stability were not found with each of the series, but the relative affinities of the three metal ions for the ligands came out as expected, Fe > Al > Cu; and the sequence of chelating strengths of the substituted salicylic acids was also as anticipated (5) in the presence of ferric and aluminum ions. This sequence is as follows: 4-amino > 4-methyl > 3,5-diisopropyl > 5-*t*-butyl > unsubstituted > 3-methyl > 5-chloro > 3-nitro > 5-nitro. With cupric ion, however, a somewhat different sequence was observed. This result pointed to a peculiarity in the reaction between salicylates and copper ion not easily explainable on steric grounds. We have since obtained both spectrophotometric and dialysis data indicating complex formation between salicylic acid and the copper-containing enzyme tyrosinase (unpublished observation). It appears that copper ion may be involved in the biological effects of salicylates.

Some results are available showing the effects of substituent groups on the therapeutic activity of salicylates and may be found in the reports of Stockman (17), Birkofer (4), and O'Brien and Thoms (14). In the last case, it was shown that *o*-thymotic acid had much greater analgesic effects that acetylsalicylic acid.

It is also noteworthy that 4-aminosalicylic acid, which has been used in the treatment of tuberculosis, appears as the most strongly bound derivative with ferric iron. A stability constant with copper ion was not obtained because of the insolubility of the copper chelate.

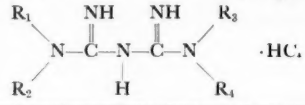
A high degree of metal-binding strength, however, is likely to prove disadvantageous for specific effects. Although chelate stabilities should probably be sufficiently great to permit the existence of the chelates in the presence of cellular chelators, such as amino acids and proteins, there is probably a limit to chelate strength for drug activity where the drug is functioning as a chelator or metal-transporting agent. Powerful chelators such as dithizone (13) can also produce anemia or other unfavorable effects. Relative weakness of binding, then, should be important where the chelating agent functions

as a transient chelator. There is some evidence suggesting that isonicotinic acid hydrazide (INH) depends on this factor for one of its biological effects, since the nature of the metal to which it is bound makes little difference in the antituberculotic activity when preformed chelates are administered to tubercular mice (9), and the administered INH chelates probably dissociate in or at the cell (6). Blocking the ability of INH to chelate metal ions has been shown to remove antituberculotic activity (7).

Both steric and electronic effects are undoubtedly of importance where a 1:1 ligand to metal ratio is required for activity. This significance has been pointed out in the case of Albert's now classical experiments on the antibacterial action of oxine (1), and is possibly operative in the diabetogenic effects of metal chelates of alloxan and related isoalloxazines (11, 12) where 1:1 chelates have been isolated and where further combination with a biologically important group is likely. Some evidence has also been found that hypoglycemic agents, β-phenethylbiguanide, may function as a 1:1 chelator with cupric ion. Tri- and tetra-substitution of the N_1 and N_5 atoms of the biguanide structure hinders chelation of copper ion and limits or removes hypoglycemic activity as well (10). This is illustrated in Table 1, where it also may be seen that the ability to chelate copper and the hypoglycemic activity are not completely parallel, so other factors must be of comparable significance. It may be significant that biguanides have been found to chelate only copper and nickel ions (10).

Factors affecting the solubility of the chelating agents or the resulting metal chelate should be considered in terms of the effect on the ratio of fat to water solubility. If the function of the chelating agent is known, whether it serves as a metal chelating or metal transporting agent, solubility could be regulated to favor the given effect. In the case of oxine, the presence of water-solubilizing and fat-repelling groups removed antibacterial effects (3). Regulation of oil-water partition coefficients proved to be revealing in regard to the antituberculotic activity of both *p*-aminosalicylic acid (8) and INH (9), as well as in regard to the antibacterial effects of a series of oxine derivatives (2). In two of these cases, it was demonstrated that increase in fat solubility of the chelates favored activity. In the case of *p*-aminosalicylic acid, the oil-water partition coefficient of the cyclic copper chelate was compared with that of an open chain copper complex of relatively high polarity. Short alkyl groups were introduced into the oxine rings to increase fat solubility of the subsituted oxine chelates. In the case of INH, measurement of oil-water partition coefficients of both 1:1 and 2:1 chelates of various metals showed the less polar 2:1 chelates to be more fat soluble, but at high dilution, over a period of time, the equilibrium shifted in favor of the 1:1 chelates. It thus pointed to the possibility of the 1:1 chelate as an entity involved in the activity against tuberculosis.

TABLE 1. *Structure and Activity of Biguanides**



Code No.	R_1	R_2	R_3	R_4	Hypoglycemic Activity	Chelate Isolated, Cu:B
DBI	$C_6H_5CH_2CH_2$	H	H	H	Good	1:1, 1:2
B_1	CH_3	CH_3	H	H	Fair	1:2
B_3	$C_6H_5CH_2$	CH_3	H	H	Good	1:2
B_4	$C_6H_5CH_2$	H	CH_3	H	Good	1:2
B_6	$C_6H_5CH_2$	H	CH_3	CH_3	Fair	1:1
B_8	$C_6H_5CH_2$	H	$C_6H_5CH_2$	H	None	1:2
B_9	$C_6H_5CH_2$	CH_3	$C_6H_5CH_2$	H	None	None
B_{10}	$C_6H_5CH_2$	CH_3	$C_6H_5CH_2$	CH_3	None	None
B_{11}	$-(CH_2)_5-$		H	H	Good	1:1
B_{12}	C_2H_5	H	CH_3	CH_3	None	None
B_{13}	n-C_4H_9	H	CH_3	CH_3	Good	None
B_{14}	n-C_5H_{11}	H	CH_3	CH_3	None	None

* These compounds and the accompanying information regarding activity were generously supplied by Dr. S. L. Shapiro, U. S. Vitamin and Pharmaceutical Corporation.

The finding of antibacterial activity in the case of metal chelates of methionine and biotin (6) can probably be explained as a fat-solubilization of metal ions which then became effective at bacterial cell walls or possibly within the cell itself. Chelating agents used for removal of toxic metal ions, such as EDTA and BAL, however, should require greater water than fat solubility to be of value.

Existing problems and unanswered questions regarding chelate reactions of drugs in biological systems may be said to be those of biological, and especially enzymatic, reactions in general. Such problems as the nature of the competition of chelating agents with cellular chelators, the state of metal ions *in vivo*, the sites of action, the relative assessment of the various factors affecting chelation, and the nature of reaction at or passage through cell boundaries will probably require different approaches and distinctive investigations to fit each system under study. The use of kinetic studies where suitable systems can be isolated or simplified may also be of value. Consideration of the factors discussed above, however, should continue to play a role in the investigation of the mechanisms by which chelating agents exert biological effects, but convincing proof of a chelation mechanism for most drugs capable of chelation will most likely be an elusive prize for some time to come.

REFERENCES

1. Albert, A., M. I. Gibson and S. D. Rubbo. *Brit. J. Exper. Path.* 34: 119, 1953.

2. Albert, A., A. Hampton, F. R. Selbie and R. D. Simon. *Brit. J. Exper. Path.* 35: 75, 1954.

3. ALBERT, A., C. W. REES AND A. J. H. TOMLINSON. *Rec. trav. chim.* 75: 819, 1956.
4. BIRKOFER, L. *Hoppe-Seyler's Ztschr. physiol. Chem.* 261: 87, 1939.
5. CALVIN, M. AND K. W. WILSON. *J. Am. Chem. Soc.* 67: 2003, 1945.
6. COUNTER, F. T., JR., R. N. DUVALL, W. O. FOYE AND R. W. VANDER WYK. *J. Am. Pharm. A.* 49: 140, 1960.
7. CYMERMAN-CRAIG, J., D. WILLIS, S. D. RUBBO AND J. EDGAR. *Nature, London* 176: 34, 1955.
8. FOYE, W. O. AND R. N. DUVALL. *J. Am. Pharm. A.* 47: 282, 1958.
9. FOYE, W. O. AND R. N. DUVALL. *J. Am. Pharm. A.* 47: 285, 1958.
10. FOYE, W. O., R. L. O'LAUGHLIN AND R. N. DUVALL. *J. Am. Pharm. A.* in press.
11. LANGE, W. E. AND W. O. FOYE. *J. Am. Pharm. A.* 45: 699, 1956.
12. LANGE, W. E., J. MCMURTRY AND M. E. AMUNDSON. *J. Am. Pharm. A.* 49: 322, 1960.
13. MCDONALD, D. F. *J. Urol.* 83: 458, 1960.
14. O'BRIEN, J. E. AND R. K. THOMS. *J. Am. Pharm. A.* 47: 258, 1958.
15. PECCI, J. AND W. O. FOYE. *J. Am. Pharm. A.* 49: 411, 1960.
16. PERRIN, D. D. *Nature, London* 182: 741, 1958.
17. STOCKMAN, R. *J. Pharmacol. & Exper. Therap.* 4: 97, 1913.
18. TURCOTTE, J. G. Master's Thesis, Massachusetts College of Pharmacy, Boston, Mass., 1960.

DISCUSSION

Design of chelates for therapeutic objectives[1]

MARTIN RUBIN

Department of Biochemistry, Georgetown University Medical School, Washington, D. C.

THOSE OF US WHO LABOR in this vineyard owe a great debt to Dr. Adrien Albert for having been one of the first to till the soil. Nor could I permit this opportunity to pass without expressing my personal debt for the inspirational guidance of his thoughts so admirably presented in his now classic monograph on "Selective Toxicity" (1).

The final test of our understanding of the normal and the diseased state, as well as our real knowledge of the structure and function of chemical compounds and their interaction with the living system, is our ability to recognize or design in advance an agent which will achieve a specific therapeutic objective. This is the opposite side of the coin from the across the board screening of compounds for as many biological parameters as possible followed then by systematic exploitation in terms of structure-function relations of the "activity" leads that have developed in the screening program. Most of us will admit that to date almost all of our useful drugs have been developed by the broad screening technique rather than the successful application of hard won knowledge to the task of rational prediction of the therapeutic utility of new compounds. The subject of Dr. Albert's talk has thus been the more difficult position to sustain. That our successes are so limited is in large measure the result of our inability in our present state of knowledge to formulate the *in vivo* milieu accurately enough to sustain the test of rational prediction. In the special case of some of the cations, however, it seems to me that our knowledge of the gross framework of transport and storage processes and the metal-binding action of synthetic chelates can permit us to venture cautiously into this dangerous territory. In the time allotted for my discussion, I propose to describe the background of the development of calcium EDTA in metal poisoning in our laboratory, to outline our thinking in the course of the development of new agents for handling a therapeutic problem of iron metabolism, to propose a path of approach for the solution of the problem of renal calcific calculi and to present some thoughts on the direction of research for the development of gold compounds for application in rheumatoid arthritis. In a sense the choice of these problems is a reflection of our increasing sophistication of knowledge of metal-binding in biological systems, as is so evident in the multidisciplined approach of this meeting.

In 1948, the inventor of EDTA, Dr. F. C. Bersworth, visited my laboratory at the suggestion of Dr. A. Lehman of the Food and Drug Administration. Dr. Bersworth had established that EDTA could inhibit rancidity development and discoloration in many foods. Since there had been little toxicological work done with the compound, it was suggested that we establish the biological actions of the compound to determine its hazard as a potential food additive. Dr. A. E. Martell was kind enough to outline the chemical nature of the compound. Our first studies on the subacute oral toxicity of the compound and its calcium chelate demonstrated (Fig 1 and Fig. 2) a relatively low order of toxicity. For the "free" EDTA,

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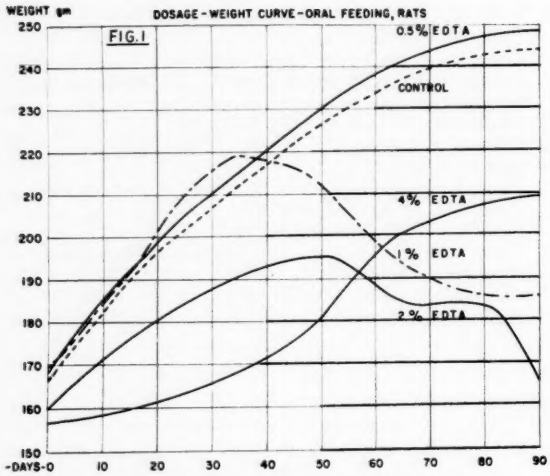


FIG. 1. Subacute toxicity of oral EDTA.

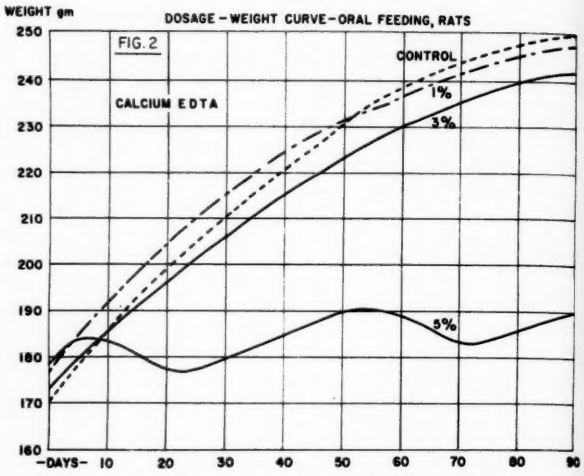


FIG. 2. Subacute toxicity of oral calcium EDTA.

n our strain of Osborne-Mendel rats on the standard aboratory diet, we found indications of toxicity at dietary levels above 0.5 per cent for a 90-day feeding period. There was no specific organ pathology. The manifestation of toxicity by weight change was, rather, related to inanition, diarrhea and dehydration. Effects of the calcium EDTA chelate *per os* were similar. Support for these conclusions was obtained by paired feeding studies (27). Subsequent two year oral toxicity studies also failed to develop indications of specific organ damage due to administration of the chelates (43). These results in our laboratory were in complete agreement with those of Fellers and his students and in accord with the more recent rat and dog studies reported at this symposium (10, 26, 30, 54).

In order to obtain more precise information about the probable biological activity of EDTA, attention was directed to parenteral administration of the compounds. The first reported animal observations (12, 13) coupled with consideration of the potential cationic chelation targets of EDTA *in vivo* suggested that hypocalcemic tetany would be the primary nature of the acute action of EDTA. The prediction was rapidly verified and, simultaneously, the relative nontoxic nature of the calcium disodium EDTA was established (33). The typical result of the injection of EDTA is illustrated in Figure 3. One may note that the oxalate precipitable calcium fraction in the blood is sharply decreased as a function of the speed, quantity and mode of parenteral administration of the compound. Rapid administration of high dosages results in death by hypocalcemic tetany. Since, at the time, the potential therapeutic utility of a hypocalcemic agent seemed limited to research study of calcium metabolism and the few clinical situations in which a sharp decrement of blood calcium might be of value (37), consideration was given to the possible medical utility of the nontoxic calcium EDTA.

We felt that human studies ultimately would be essential to clearly delineate the hazard involved in the proposed use of the compound as a food additive. It seemed reasonable to expect that the development of a valid therapeutic use for calcium EDTA might lead to human data. Calcium EDTA had been found as effective as the parent compound in food applications by virtue of the ability of cations of higher binding strength to displace the calcium and form the corresponding catalytically inert metal chelate. The *in vitro* evidence of this metal displacement reaction (32) raised the possibility that the same process might occur *in vivo*. Suitable animal studies rapidly established the curative value of calcium EDTA for cobalt and nickel poisoning in rats with an evident decrease in the acute toxicity of these metals between thirty- to fiftyfold when administered as the EDTA chelates (38). In contrast, clear evidence of the efficacy of calcium EDTA treatment in acute lead poisoning in experimental animals was not readily attained in our hands (40). Curiously, the inconclusive results in calcium EDTA treatment of experimental acute lead poisoning occurred despite a readily demonstrable increase in urinary lead excretion induced by the compound (Fig. 4). On the other hand, experimental acute lead poisoning in animals unfortunately does not simulate the disease process in the human. It is evident from the graph that each treatment of calcium EDTA results in a sharp enhancement in urinary lead excretion. In the clinical situation, the toxic manifestations of the metal usually become evident after a relatively long period of low level ingestion. Treatment had been based on measures designed to clear the metal from the areas of potential toxicity in blood and soft tissue by redeposition of lead in bone. Administration of calcium salts had been thought to achieve this objective (49).

Even though calcium EDTA was not highly effective in the acute toxicity of experimental lead poisoning, the nonidentity of the animal studies with the clinical course of lead poisoning, coupled with the proven enhancement of urinary lead excretion by calcium EDTA and the nontoxic nature of the drug, led us to attempt clinical

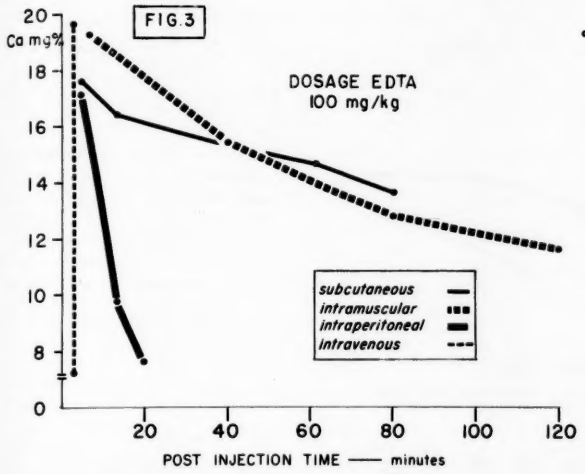


FIG. 3. Effect of EDTA on blood calcium.

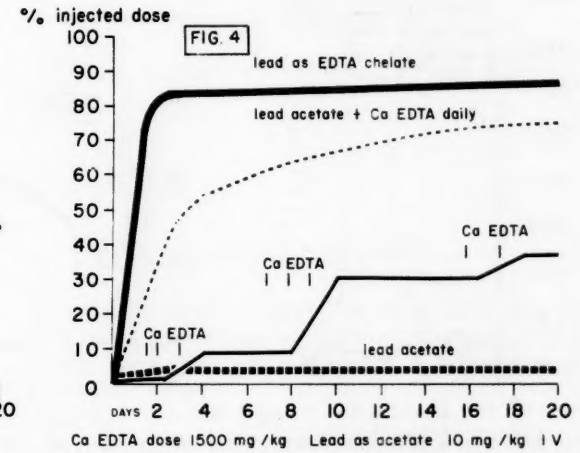


FIG. 4. Effect of calcium EDTA on urinary lead excretion in rabbits.

trial of the compound in a severe case of lead poisoning in a child (6). The indication of favorable response prompted our further study in the field (7, 39) with rapid development of confirmatory evidence of the value of the drug in this condition. Others, of course, have notably extended our studies (17, 20, 23, 35). Recital of this background may serve to provide an explanation for Dr. Seven's comment, ". . . an interesting jump in the usual evolutionary process occurred when the parent compound EDTA and its related compounds passed into the hands of clinicians after a relatively short period of basic laboratory evaluation" (47).

In summary, I would suggest that the present application of calcium EDTA in lead poisoning provides an example of the recognition of physical and chemical properties of a compound which led to its deliberate test and final application in a preselected area of therapy.

Having completed this demonstration, it was to be expected that the question would be asked as to why the agent might not also be applicable in other metal poisonings. Following a suggestion by the late Dr. Ephriam Shorr (48), in collaboration with Dr. J. V. Princiotto of this University, we proceeded to test the applicability of calcium EDTA in iron storage disease, a process of the long term excess absorption and tissue deposition of iron. We could demonstrate little enhancement in iron excretion in this state with calcium EDTA. The same result was reported by others (53). The availability of N-hydroxyethylethylenediaminetriacetic acid (HEDTA, HOEDTA) and reports that the compound had superior iron binding ability compared to EDTA (8) led us to examine its toxicity and then its applicability in iron storage disease. Although the results showed some improvement over those with EDTA, they could not be considered highly significant as a potential form of treatment (46). As one effort to direct the compound more precisely to areas of iron deposition in the disease state, we prepared various esters of HOEDTA and tested the ethyl ester, especially, for applicability in iron removal. The rationale for this approach was based on the expectation that, while the esters themselves might be ineffective as iron chelating agents, they would be absorbed from areas of local injection, transported to the liver and then converted by esterase activity to the free and effective iron binding agent at a place of pathological iron accumulation. While the animal results supported the hypothesis in showing a marked enhancement in urinary iron excretion (Fig. 5) (11), the compound turned out to be too toxic for clinical applicability (47). These more obvious approaches to the problem having had only limited success, it seemed clear that a more intensive study of the interrelation of chelates and iron metabolism would be justified.

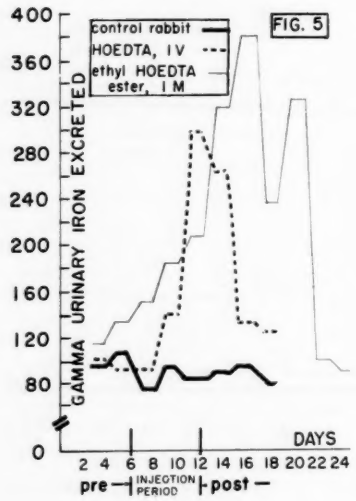
Dr. Eichhorn has pointed out the wide variety of iron binding compounds which occur *in vivo* (14). In addition, the problem of controlling the movement of iron is complicated greatly by the dynamic forces that influence iron in the body. This subject has been recently reviewed in some detail (52). A point of considerable significance is the fact that no physiological mechanism exists for the excretion of iron. A useful therapeutic agent would be required to accept iron at areas of pathological deposition and transport the metal to the excretory channel. Thus, the compound would have to fulfill the following requirements:

a) It would have to be nonmetabolized in order to proceed to the excretory channels.

b) Its iron binding kinetics and strength should serve to permit the rapid formation of the iron chelate at storage areas and its preservation in the *in vivo* milieu.

c) The iron chelate when once formed should be capable of complete excretion at all quantitative levels.

The studies of Foreman *et al.* (18, 19) for EDTA, as well as our own for some other members of the amino



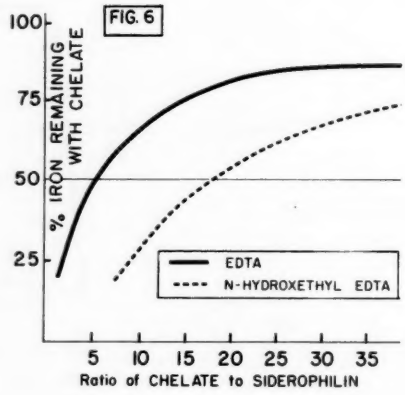


FIG. 5. Effect of N-hydroxyethylethylenediaminetriacetic acid (HEDTA, HOEDTA) and its ethyl ester on urinary iron.

FIG. 6. Distribution of iron between specific iron binding protein (IBP, siderophilin) and chelates of EDTA and N-hydroxyethylethylenediaminetriacetic acid.

acid chelate group, permitted the conclusion that this class of compounds met the requirements for metabolic inertness (29, 51) and a high degree of urinary excretion. By a series of competitive binding studies in plasma, we could, in addition, demonstrate that compounds of the order of iron binding strength of EDTA were able to hold the iron atom in the presence of even so potent an iron binding agent as the specific iron binding protein (IBP, siderophilin) of the plasma (41) (Fig. 6). One may note that the shift in mole ratio of chelate to IBP results in competitive movement of the metal to the synthetic ligand as a function of its increasing mole excess. This behavior would be expected for compounds of about equal binding strength, as has been pointed out by Dr. Reilley in this symposium (36). Ferric EDTA has a log stability value of about 10^{26} (9). According to Dr. Saltman's statement, the measured value for the second iron atom of IBP is about 10^{27} (45). Since our own experiments relate to the competitive binding of the second iron atom of IBP, the agreement on the subject is striking.

It is of interest that the question of the iron binding strength of the compound is by no means the only point involved in the determination of whether iron will move from the chelate to other sites of attachment *in vivo*. Since this may be of some interest, I would like to present some data from recent studies. The reticulocyte has been shown to take up iron from the environment when the iron is presented in the form of the iron binding protein. Iron presented to the cell in the form of the EDTA chelate was reported as unavailable for incorporation into hemoglobin (4, 22). In further study of this system, we have been able to show that the uptake of iron into the cell is a function of the molecular architecture of the transporting agent as well as its intrinsic iron binding ability. In Table 1, one may note that for a series of compounds of closely related structure such as I, II, III and IV (Fig. 7) the transition from a lipotropic series of compounds I, II and III to the hydrophilic structure as in the sulfonic acid derivative IV results in marked decrease in the ability of the compound to deliver iron to the cell. One may note also that the highly hydrophilic EDTA and DPTA also result in minimal iron transport. It should be pointed out that these studies were conducted with physiological levels of iron in the medium, that they are on cells in suspension rather than the more complex slices, and finally that the iron has been swamped by excess chelating agent to insure that the results relate to a homogeneous chelate species. The data have marked analogy to those reported by Albert *et al.* (2) for the cellular transport of 8-hydroxyquinoline and its derivatives. Albert found that the anionic sulfonic acid of 8-hydroxyquinoline, although of comparable chelation ability to other lipotropic compounds in the series, was excluded from the bacterial cell.

We conclude from this type of evidence that the iron binding strength of the carrier is only one determinant of the movement of transported metal. The molecular structure may be of equal moment in the outcome of the process. Certainly, if we were to hope to cause the *in vivo* removal of iron from tissue depots, we would search in the family of the highly polar hydrophilic structures. In this way we would hope to chelate iron at cell surfaces to permit its transport through the water compartment. EDTA meets all the requisites for a useful compound yet fails to achieve significant clinical success. Study of the distribution and excretion of Fe^{59}-tagged EDTA demonstrated that it was subject to the operation of a threshold effect *in vivo*. At low dosage levels the iron was retained by the organism while at high levels of parenteral administration up to 70 per cent of injected iron was excreted in the urine (31). This relation is a common one for many metals. It results from the variability of the strengths of the many metal-binding sites which are known to be present in the organism. Only after the potent iron binding areas are saturated is it

TABLE 1. *Reticulocyte Iron Uptake*

Compound	Surface	Intracellular
IBP	3.6*	4.6*
I	2.0	3.8
II	2.9	4.3
III	3.2	6.1
IV	0.1	0.1
EDTA	0.1	0.1
DTPA	0.1	0.1

* Percentage of total Fe^{59}.

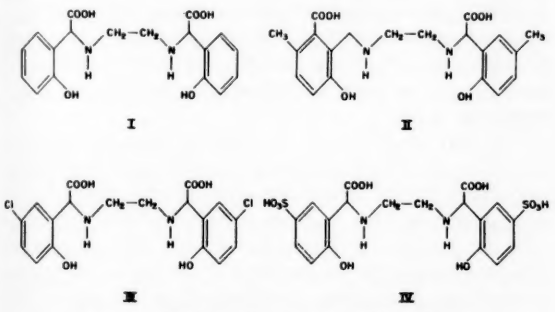


FIG. 7. *o*-Hydroxyphenylacetic acid chelates.

possible for EDTA to escort iron to the urine. The quantitative binding relation between iron, EDTA, and some sites *in vivo* is not favorable enough for iron EDTA to result in marked increase in urinary iron excretion in iron storage disease.

In their studies on the metabolism of carbon-tagged EDTA Foreman *et al.* (19) observed that a small fraction of the compound remained trapped in the body. They suggested that this might be the product of iron fixation, a thought which is compatible with present results. In the case of DTPA, however, we could demonstrate that excretion of the iron complex was quantitative even when the administration was in trace quantities. We may thus anticipate that for this compound the formation of the iron chelate *in vivo* would result in its complete clearance in the renal system. Since DTPA fitted, best of all the agents available, the criteria we had established for the induction of iron excretion in iron storage disease, it was subjected to clinical trial. This work was carried out by Dr. Charles Rath and his colleagues of this institution. A test dosage of 2.5 gm of calcium DTPA evoked a urinary excretion of up to 60 mg of iron in a 24-hour period. Additional studies have shown that the agent may cause the excretion of a significant amount of iron in the urine (15, 16). In Table 2, we have presented a series of patients with varying degrees of tissue iron and the urinary iron excretion following a test dose of calcium DTPA. Of interest is the observation that the amount of urine iron excretion parallels the extent of the tissue iron. Thus we have here an agent which exerts its therapeutic action in accord with the demands of the disease process. When pathological iron deposition is great, iron excretion is high. In the normal patient treatment does not deplete iron stores. In a sense this is a direct example of the process of selective toxicity.

TABLE 2. *Correlation of Iron Stores and Urinary Iron After Calcium DPTA*

Patient	Estimated Body* Iron Stores	Urinary Iron (mg) After 2.5 gm Calcium DTPA
W. D.	4+	42.0
P. M.	4+	30.4
P. H.	3+	22.4
R. D.	2+	7.0
H. D.	2+	2.4
W. W.	1+	0.3
L. D.	0	0.8

* By marrow examination and liver biopsy.

The above account is illustrative of the preselection of an agent for a given disease process and the nature of the modifications that have been required to achieve even a reasonable degree of success in the attainment of the objective.

May I suggest another problem which seems to us to be ready for rational solution by design of an agent for a specific therapeutic objective? A large proportion of renal calculi are alkaline earths in their primary composition. We all realize that the solubilization of calcium and magnesium concretions is an area of successful chelate application in many industrial usages (9). Early in the development of medical applications of chelating agents it was possible to demonstrate *in vitro* and *in vivo* that an alkaline earth calculus could be dissolved, for example, by immersion in or retrograde irrigation by EDTA solutions (24, 34). It was of interest that the known complexity of the multilayered organic structure of the urinary calculus did not appreciably inhibit the dissolving ability of the chelate solution. Unfortunately, two problems have forced this promising approach to be abandoned. In the first place, the chelate solutions introduced into the kidney have been highly irritating leading to bleeding and severe pain. Secondly, it is an unfortunate fact that solutions introduced by the retrograde route do not come effectively in contact with a stone located in the pelvis or body of the kidney. Were it available, the ideal agent should be absorbable by mouth, pass through the body unchanged, and then be excreted in the urine in such a form as to be able to either dissolve alkaline earths or to maintain them in solution for purposes of therapy or prophylaxis in the appropriate clinical circumstances.

The amino acid chelates in general fit the requirement that the compounds move through the body unaltered and are excreted in the urine. It is within the range of reasonable expectation to hope that suitable modifications in dosage formulation would provide compounds that could be absorbed from the gastrointestinal tract in order to mitigate the present problem of parenteral administration. Chelate esters, for example, might follow lipid absorption pathways to be cleaved in the liver for conversion to the active chelate form of the drug for urinary excretion. The basic problem is the paradoxical one that the successful compound needs to be one which will traverse the magnesium and calcium laden body fluids and yet be excreted into the kidney in a

calcium and magnesium free form, able there to bring the alkaline earths into solution. This is indeed an application of selective toxicity in the broad sense of the term since we are requiring that the chosen agent proceed through a predetermined path to select a given target area from others, perhaps even more inviting in the same environment.

Two approaches to the problem have thus far suggested fruitful avenues of study. Starting with the assumption that an animal acutely deprived of calcium would institute all possible physiological mechanisms for the conservation of the element, we have endeavored to determine whether we could induce the organism to abstract calcium from a chelate just prior to the passage of the agent and its alkaline earth burden into the urine. In this event, for example, a compound such as calcium EDTA would move through the body to the kidney where the calcium would be left behind and the chelate would continue on into the urine in a form ready to pick up a fresh burden of the alkaline earth from a calculus. To test this possibility, we first established the conditions under which we could routinely establish an acute hypocalcemic state in experimental animals (3). Figure 8 demonstrates the time response in blood calcium level in the adult rat to a standardized nonlethal dosage of EDTA. Under these conditions, the restoration of the depressed blood calcium level requires a period of almost 24 hours before normality is achieved.

It is of passing interest that studies of this calcium restoration reaction have shown significant variation as a function of the state of calcium metabolism of the animal. Young normal animals respond differently than normal adults. Rachitic animals and parathyroidectomized animals in turn have shown altered blood calcium restoration curves compared to normals. The concept that the bone may serve as an almost inexhaustable reservoir of calcium for homeostatic maintainence of blood calcium levels stems from two lines of evidence. On the one hand, experiments such as those of Hastings and Huggins, who bled, decalcified and retransfused the decalcified blood to a dog, demonstrated the sharp rapidity of blood calcium level restoration (21). On the other hand, the graphic visualization of the bone as a tremendous surface expanse has provided a perhaps too simple picture of the facile physico-chemical equilibration of the ambient liquid and the solid bone phase (28). These concepts may serve as a satisfactory support for much of our thinking on calcium metabolism, but the use of calcium binding chelates has served to help set limits on the operative range for these homeostatic mechanisms. Thus we may observe that the blood calcium restoration following EDTA hypocalcemia does have a dosage response relation that is not unlimited. It is the response at maximum tolerated dosage which provides the most useful comparative information regarding the animal's calcium metabolic status.

We may inquire into the fate of injected calcium EDTA in an animal subjected to this type of hypocalcemic stress. Whereas in the normal animal some 70 per cent of the injected calcium is excreted into the urine with its chelate carrier, in the animal with low blood calcium levels only 33 per cent of the injected calcium reaches the urine with the chelate (42). Thus, by this drastic experimental procedure, we have been able to induce the chelating agent to part company with the calcium at the kidney level, as it turns out by further investigation. What has been done with animals also has been achieved in the human. Spencer *et al.* caused a decrease in elevated blood calcium levels in a hyperparathyoid patient by the injection of EDTA. In this patient the urinary calcium excretion then decreased from 92 per cent pretreatment to 30 per cent posttreatment of the values expected from the EDTA level in the urine (50). Thus, in both animals and humans, we find that it is possible to so direct the course of a chelate molecule through the mineral laden body so that it ends up in the urinary excretion largely free of calcium or magnesium. Although this technical solution of the problem has yielded much information of theoretical interest in calcium metabolism, it is without practical value for the specified clinical objective. It is not possible to maintain the abnormally hypocalcemic state for the

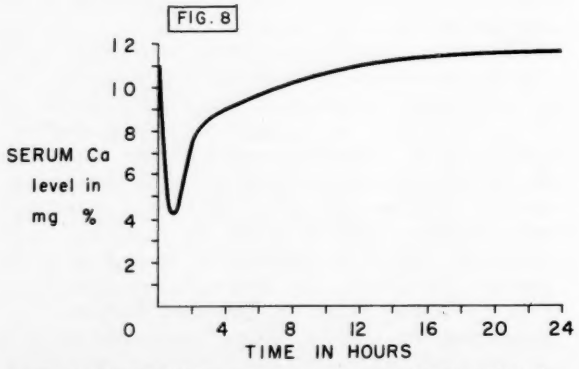


FIG. 8. Blood calcium response to intraperitoneal EDTA in rats, 0.6 mM/kg.

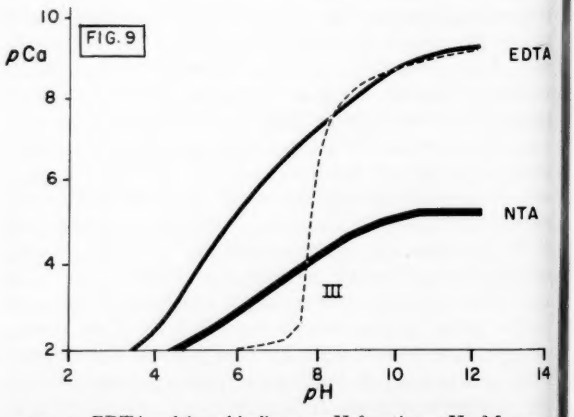


FIG. 9. EDTA calcium binding as pH function. pH-pM curves for calcium chelation. (See text.)

necessarily long time periods required for this slow therapeutic procedure. For this reason, we have directed our attention to a second approach to the problem.

Dr. Reilley has summarized ably the relation between chelate binding of alkaline earths and the pH of the medium. In the case of EDTA, as is demonstrable *in vivo*, EDTA is essentially saturated with calcium in its passage through the body and one can expect little gain in calcium solubilization by this agent by the elevation of pH from the systemic 7.4 to a possible urinary pH of 8.0. A more satisfactory agent would be one whose pM-pH characteristics were those of curve III (Fig. 9). Such a compound could be expected to pass through the body at pH 7.4 without calcium binding and then be available in a more alkaline urine for maximal solubilization of alkaline earths.

Is it possible to design a compound that would possibly fill these criteria? For the pH range under consideration, it seems evident that the aminocarboxylic acid types alone, at this time, offer the requisite degree of alkaline earth binding. In this group of compounds, the permissible range of structural variability with retention of potent binding ability is narrow.

Some of the essential steric and structural factors requisite for maximal alkaline earth binding have been recently described (25). It has been pointed out also that the slope of the pM-pH curve is dependent on the base strength of the metal-binding ligands as well as on the number of dissociable protons in these groups (9). For the system under consideration, we feel that the ideal compound should have the steric alignment most suitable for alkaline earth chelation as, for example: fixed geometric relation of the nitrogen atoms; molecular substitution by electron repelling groups to increase the base strength of the nitrogen atoms; suitable substitution around the carboxyl groups to decrease their acidity; as large a number of dissociable protons as possible in the molecule; and also an overall structure which will withstand metabolic destruction in passage through the body. Our efforts have been directed along lines which we hope will attain these goals.

Finally, I would suggest one other area for which the tools seem to me to be at hand for the prediction of structural requirements for therapeutic utility. Present treatment of the inflammatory processes of rheumatoid arthritis rests on the use of the corticosteroids. It is agreed generally that while these agents are effective suppressors of the inflammatory process they do not alter the course of the underlying disease. In addition, it is still true, despite the impressive advances that have been made in enhancing anti-inflammatory action while minimizing other hormonal effects, that a significant number of serious side effects in the patients restrict the full and free use of this important group of potent agents. Prior to the use of corticoids, and even now to a certain extent, gold compounds have had wide application in treatment of rheumatoid arthritis. When used early in the course of the disease process, there has been some suggestion that gold may exert a curative rather than solely a suppressive action. The disadvantages of gold therapy have been notable, however, in that once introduced into the body the metal is fixed for long periods. For this reason, the onset of toxic manifestations in a person carrying a large body burden of gold can not be readily ameliorated by removal of the metal. Toxicity manifested by skin rashes, liver disease, and bone marrow involvement may be severe enough for fatal outcome. An improvement in gold therapy would be possible were a carrier devised which would permit ready excretion of the gold not required for the therapeutic result. Such a compound would permit treatment in the manner in which digitalis is used in cardiac problems. Maintenance therapy would be possible with rapid decrease in the gold level at the onset of toxic symptoms.

The stereochemistry of the tetracovalent Au^{+3} complexes suggests that a suitable compound for gold therapy would be one in which sulfur and nitrogen atoms could be fixed in a square coplanar structure (5). In such a molecule, the addition of highly polar solubilizing groups would confer a degree of water solubility to permit free distribution and excretion in the water space of the body. In addition, the hydrophilic groups should contribute to the exclusion of the molecule from the intracellular compartments. One would rely on the spacing between the sulfur and nitrogen atoms to provide the degree of complex stability for the gold which would allow its cleavage at the site of therapeutic need. This could be at either the area of inflammation or at whatever site *in vivo* present gold compounds exert their biological action. A compound of this type would be exerting selective toxicity in either delivering gold to the area of use or causing it to be eliminated in the urine. Such a material should have high therapeutic efficiency and low toxicity. At this time, we are attempting to test these hypotheses in the laboratory.

REFERENCES

1. ALBERT, A. *Selective Toxicity*. New York: Wiley, 1951.
2. ALBERT, A., S. D. RUBBO, R. J. GOLDACRE AND B. G. BALFOUR. *Brit. J. Exper. Path.* 28: 69, 1947.
3. ALEXANDER, R. Ph. D. Thesis. Georgetown Univ., Wash., D.C., 1959.
4. ALLEN, D. W. AND J. H. JANDL. *Blood* 15: 71, 1960.
5. BAILAR, J. C., JR., (editor). *The Chemistry of the Coordination Compounds*. New York: Reinhold, 1956, p. 371.
6. BESSMAN, S. P., H. REID AND M. RUBIN. *M. Ann. District of Columbia* 21: 312, 1952.
7. BESSMAN, S. P., M. RUBIN AND S. LEIKIN. *Pediatrics* 14: 201, 1954.
8. CHABEREK, S. AND A. MARTELL. *J. Am. Chem. Soc.* 77: 1477, 1955.
9. CHABEREK, S. AND A. E. MARTELL. *Organic Sequestering Agents*. New York: Wiley, 1959, p. 192.
10. CHAN, M. S. Ph. D. Thesis. Univ. of Mass., Amherst, Mass., 1956.
11. DEEB, G. Master's Thesis, Georgetown Univ., Wash. D.C., 1957.
12. DYCKERHOFF, H., R. MARY AND B. LUDWIG. *Ztschr. ges. exper. Med.* 110: 412, 1942.
13. DYCKERHOFF, H., R. MARY AND B. LUDWIG. *Ztschr. ges. exper. Med.* 113: 291, 1944.

14. Eichhorn, G. This Symposium.
15. Fahey, J., I. Brick, J. V. Princiotto, C. Rath and M. Rubin. *J. Lab. & Clin. Med.* 57: 436, 1961.
16. Fahey, J., J. V. Princiotto, C. Rath and M. Rubin. *Circulation Res.* 3: 52, 1960.
17. Foreman, H., H. L. Hardy, T. L. Shipman and E. L. Belknap. *A.M.A. Arch. Indust. Hyg.* 7: 148, 1953.
18. Foreman, H. and T. T. Trujillo. *J. Lab. & Clin. Med.* 43: 55, 566, 1954.
19. Foreman, H., M. Vier and M. J. Magee. *J. Biol. Chem.* 203: 1045, 1953.
20. Hardy, H. L., H. B. Elkins, B. P. W. Ruotolo, J. Quimby and W. H. Baker. *J.A.M.A.* 154: 1171, 1954.
21. Hastings, A. B. and C. B. Huggins. In: *Metabolic Interrelations.* New York: Josiah Macy, Jr. Foundation, 1951, p. 38.
22. Jandl, J. H., J. K. Inman, R. L. Simmons and D. W. Allen. *J. Clin. Invest.* 38: 161, 1959.
23. Karpinski, F. E., F. Rieders and L. S. Girsh. *J. Pediatrics* 42: 687, 1953.
24. Kissin, B. and S. Natelson. *Science* 112: 367, 1950.
25. Kroll, H. and M. Gordon. *Ann. New York Acad. Sc.* 88: 341, 1960.
26. Krum, J. K. Ph. D. Thesis, Univ. Mass., Amherst, Mass., 1948.
27. Larson, R. H. Ph. D. Thesis, Georgetown Univ., Wash. D.C., 1958.
28. Neuman, W. In: *Metabolic Interrelations.* New York: Josiah Macy, Jr. Foundation, 1954, p. 39.
29. Orzell, R. Master's Thesis, Georgetown Univ., Wash. D.C., 1957.
30. Oser, B. This Symposium.
31. Patchman, E. Ph. D. Thesis, Georgetown Univ., Wash. D.C., 1959.
32. Plumb, R. C., A. E. Martell and F. C. Bersworth. *J. Phys. Colloid. Chem.* 54: 1208, 1950.
33. Popovici, A., C. F. Geschickter, A. Reinovsky and M. Rubin. *Proc. Soc. Exper. Biol. & Med.* 74: 415, 1950.
34. Raymond, S. and R. F. Gehres. *Proc. Soc. Exper. Biol. & Med.* 74: 715, 1950.
35. Reiders, F., W. G. Dunnington and H. Brieger. *Indust. Med.* 24: 195, 1955.
36. Reilley, C. N. This Symposium.
37. Rubin, M. In: *Metabolic Interrelations.* New York: Josiah Macy, Jr. Foundation, 1954, p. 344.
38. Rubin, M. and G. Di Chiro. *Ann. New York Acad. Sc.*. 78: 764, 1959.
39. Rubin, M., S. Gignac, S. P. Bessman and E. L. Belknap. *Science* 117: 659, 1953.
40. Rubin, M., S. Gignac and A. Popovici. Abstract of Papers, 121st Meeting Am. Chem. Soc., April 1952, p. 3J.
41. Rubin, M., J. Houlihan and J. V. Princiotto. *Proc. Soc. Exper. Biol. & Med.* 103: 663, 1960.
42. Rubin, M. and G. Lindenblad. *Ann. New York Acad. Sc.* 64: 337, 1956.
43. Rubin, M., A. Martell and F. C. Bersworth. *The Biological Actions of the Versenes.* Versenes, Inc. (Dow Chem. Co.), 1954.
44. Rubin, M. and J. V. Princiotto. *Ann. New York Acad. Sc.* 88: 450, 1960.
45. Saltman, P. This Symposium.
46. Seven, M. J., H. Gottlieb, H. L. Israel, J. C. Reinhold and M. Rubin. *Am. J. M. Sc.* 228: 646, 1954.
47. Seven, M. J., In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p 223, p. IX.
48. Shorr, E. In: *Metabolic Interrelations.* New York: Josiah Macy, Jr. Foundation, 1954, p. 350.
49. Sollmann, T. *A Manual of Pharmacology* (7th ed.). Philadelphia: Saunders, 1948, p. 992.
50. Spencer, H. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 104.
51. Swantko, J. Master's Thesis, Georgetown Univ., Wash. D.C., 1957.
52. Wallerstein, R. O. and S. R. Mettier. *Iron in Clinical Medicine.* Berkeley: Univ. California Press, 1958.
53. Wishinsky, H. T., T. Weinberg, E. M. Prevost, B. Burgin and M. J. Miller. *J. Lab. & Clin. Med.* 42: 550, 1953.
54. Yang, S. S. Ph. D. Thesis, Univ. Mass., Amherst, Mass., 1952.

GENERAL DISCUSSION

Dr. Paul Saltman (University of Southern California, Los Angeles): We have found that iron suitably complexed with carbohydrates or polyols in a low molecular weight, uncharged form moves directly from the lumen of the gut into the blood. From the work in our laboratory and in the laboratories of several other investigators—Cartwright, Heilmeyer, Elmer Brown, and others—it appears that the story of ferritin as the mandatory agent for the facilitated diffusion of iron is pretty well excluded. If we plot the amount of radioactivity in the blood versus time, the moment that you put the iron into the gut either in the ferric or the ferrous state—and it is important to realize that we have isolated the iron compound following injection and find no change in the oxidation state—this iron will move directly into the blood and reach a maximum level within 90 minutes. The rate of transport into the blood seems to be a direct function of the size and charge of the chelate, but it is also a function of the degree of saturation of the iron binding capacity of transferrin.

We can demonstrate this in several ways. We can correlate the rate directly with the experimentally determined unbound iron binding capacity. We can also use the rabbit preparation which is rapidly taking up iron from the gut and inject non-isotopic iron intravenously to saturate the iron binding capacity within a very short time. The uptake of the isotope from the gut stops within a few minutes. Such experiments lead us to propose a new hypothesis which is presented in Figure 1.

If we consider the entry of iron from the lumen into the cell of the wall, the critical regulatory mechanism is the chemical state of the complexes of the metal. This is particularly important when we consider the nutritional availability of iron. If there were a "mucosal block" as proposed by Granick (Granick, S. *Physiol. Rev.* 31: 489, 1951), and as it now appears in every textbook, you would never expect to see a pathological condition like Bantu cytosiderosis where the tremendous amount of dietary iron presented to the organism is continuously taken up by the tissues. We have been able to demonstrate the rapid deposition of iron in the liver and spleen of rats fed suitable chelated forms of iron. The iron that moves into the cell enters into a spectrum of equilibrium binding reactions with sites on proteins, nucleic acids, and small molecule chelates such as organic and amino acids. Whether or not the iron moves out of the cell and into the blood as the

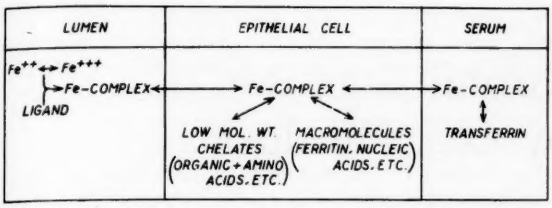


FIG. 1. Schematic representation of hypothesis of iron metabolism from the gut.

same chelate which entered, we cannnot say. Once in the blood another equilibrium condition is established with transferrin and other available agents. Uniquely, some of the new chelates that we have developed can be excreted rapidly in the urine. It appears that the chelate enters the blood from the lumen of the gut and then is able to cross the kidney membranes. We are carrying out experiments to label both the iron and the chelating agent to observe if there is parallel movement of metal and ligand.

DR. ELMER B. BROWN (Washington School of Medicine, St. Louis): I can't say that ferritin is not present at all in the mucosal cells but certainly when you look in the mucosal cells in species other than the guinea pig you have a hard time finding ferritin. I have looked at the dog and at the rat, and though I have not used the extremely sensitive assay techniques with the antiferritin antibodies, it is very difficult to find any evidence of ferritin in the mucosal cell. Practically all of the studies of Granick and of Heilmeyer have been done in the guinea pig, which may be a rather unique species.

DR. METZLER: I feel that the possibility of polymerization must always be seriously considered in describing soluble chelates. I'd like to mention an example of specificity observed with riboflavin chelates. We attempted to study the formation of complexes of flavins with cupric, zinc and nickel ions but could not obtain any evidence by titration or spectrophotometry for soluble complexes of flavins with these metals. So we turned to something which looked simple—the riboflavin-silver complex which has a bright red color and is soluble. In this case we had no difficulty in demonstrating that a proton was released by the binding of the silver. This proton must come from the dissociable hydrogen atom of the isoalloxazine ring, since lumiflavin forms a similar red complex but 3-methyl-lumiflavin does not. This coloration is highly specific. Only silver and cuprous copper of many common metals tested give the color. A second interesting fact is that the colored product is polymerized. Several observations suggested this fact, and it was confirmed by finding that the colored material gradually sediments in an ultracentrifuge. At present there seems to be no evidence that flavins form soluble monomeric chelates.

DR. SCHUBERT: When evaluating chelating agents under physiological conditions one must subtract the calcium binding (Schubert, J. *Ann. Rev. Nuclear Sci.* 5: 369, 1955). For example, in the table of Dr. Albert, the formation constant of glycine in log units is 9 while that of EDTA is 19. Actually glycine is about as strong as EDTA because the value of the ratio log K (Cu)/(Ca) is about 8 for each. Therefore, when comparing chelating agents for biological systems, one must compare the values of log K (M)/(Ca) rather than absolute values since one always has an exchange of calcium in the biological system with the particular metal.

Dr. Chenoweth mentioned that in designing a drug, the strongest chelating agent isn't always the best. A few years ago I pointed out that the biological activity of a drug plotted against the log K of the chelating agent can be assumed to have a bell-shaped curve (Schubert, J. *Chimia (Swiss)* 11: 113, 1957) for those drugs which form a ternary complex: enzyme-metal-drug. This concept led to the conclusion that the optimum activity of the metal will come about when the binding of the metal for the drug is equal to the binding of the metal for the enzyme it acts upon. Hence, depending on which side of the bell-shaped curve the formation constant happens to lie, an increase in the binding strength as the drug structure is changed, could lead to an increase or decrease in biological activity. Thus, one can alter the formation constant of a drug by making slight changes in structure or by inserting simple electron accepting or donating groups into the molecule. A plot of the resulting biological activity against the formation constant would enable one to estimate the optimum value of the formation constant.

DR. CHENOWETH: Dr. Albert, would you like to comment on the calcium subtraction?

DR. ALBERT: I think we learned many useful things from Dr. Schubert's paper in *Chimia* which I am relieved to see I quoted in my manuscript. I quite like this idea of calcium subtraction and have tried a few calculations with it. While on this general topic, I wonder if at the present time we are not concentrating too much on aliphatic chelating agents—not that it wasn't formerly wise to do so—but their possibilities are getting exhausted. Where biologists want to keep chelation extracellular why do they not try oxine sulfonic acid which has high chelating ability? It doesn't enter cells and has extremely low toxicity. Also, although I have not seen any references to them, shouldn't consideration be given to phenanthroline sulfonic acid and dipyridyl sulfonic acid?

Observations on the chronic ingestion of a chelating agent by rats and dogs

BERNARD L. OSER

Food and Drug Research Laboratories, Inc., Maspeth, New York City

DETERIORATIVE CHANGES IN FOODS AND BEVERAGES such as the development of off-flavor, discoloration or turbidity, and oxidation of certain vitamins, are often induced or catalyzed by metallic ions, particularly iron and copper. The use of chelating agents or other sequestrants to "trap" these elements in non-ionic, soluble form has proved of considerable interest to food technologists. Among the most potent of the chelating agents from the standpoint of the small concentration needed for effective results are ethylenediaminetetraacetic acid or ethylenedinitrilotetraacetic acid (EDTA) and its salts. However, in order to meet the requirements for safety under conditions of use it was necessary to conduct extensive studies in animals with a commercially reproducible product of sufficient purity for food use. The calcium disodium salt,[1] being least likely to interfere with calcium utilization, was selected for this purpose.

Acute and two-year feeding studies in rats and one-year studies in dogs were conducted with calcium EDTA. After preliminary acute toxicity studies and range-finding experiments to establish a dosage level at which an effect could be induced in rats, two-year feeding studies in rats (25 males and 25 females per initial dosage level) and one-year studies in dogs (4 per dosage level) were undertaken. The observations made in the parent generation of rats and in three successive generations included growth, food utilization, reproduction, lactation, physiological behavior, and hematology and hemochemistry as related particularly to hematopoiesis and metalloenzymes. Gross autopsy examinations and histopathological studies were also conducted of all major organs and tissues. Except for reproduction studies, similar observations were made in the dogs.

As a result of these extensive studies it was concluded that the safety of EDTA in amounts required for application in food processing could be established. The detailed data are filed with the Food and Drug Administration in support of a food additives petition for a regulation permitting the use of calcium EDTA in various foods and these data will be published in a scientific journal in due course.

[1] Versene CA, supplied by the Dow Chemical Company, Midland, Michigan.

DISCUSSION

Effect of metal-binding agents on metalloproteins[1,2]

W. W. WESTERFELD

Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, New York

IN ANY CONSIDERATION of the potential biological effects from the chronic administration of chelating agents, two factors should be considered: 1) will the chelator remove a trace element from its natural biological site and

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[2] The following abbreviations are used: EDTA (ethylenediaminetetraacetic acid); OP (1,10- or *o*-phenanthroline); BP (α,α-bipyridyl); DDC (diethydithiocarbamate); 8HQ (8-hydroxyquinoline); BAL (2,3-mercaptopropanol); SH (sulfhydryl); GSH (glutathione); cyto c (cytochrome c); MB (methylene blue); XO (xanthine oxidase); chemical symbols for metals and ions.

thereby produce a deficiency of that element; and *2*) if the chelator does not remove a trace element, will it still combine with that element *in situ* and thereby inhibit its normal physiological function? Generalizations will be difficult if not impossible because different chelators may affect any given enzyme differently, and may cross membrane barriers with varying facilities. Moreover, the normal dynamic replacement of an enzyme *in vivo* might provide opportunities for interference by a chelator which could not be detected by studies of the intact enzyme. Nevertheless, some idea of metal-protein stabilities in relation to chelators can be obtained from a survey of the *in vitro* effects of metal-binding agents on known metalloenzymes, transport and storage forms of the metal.

Iron, Cu, Mn, Zn, and Mo have clearly been established as essential micronutrients for both plants and animals. The need for Mo in animal nutrition has been established only by the use of tungstate as a competitive inhibitor (94). In addition to the above, iodine and cobalt have specialized functions in animal nutrition, as related to thyroxine and vitamin B_{12}, respectively, without having any known function in plants. Conversely, boron and vanadium are well established requirements for plants without having any recognizable need by animals. Selenium (209, 213, 232, 233) and chromium (234) have biological effects in animals which indicate a possible essential function, but too little is known about their biochemical functions to consider them here. The possibility of other trace elements playing an essential role in animal physiology has been reviewed recently (145, 231, 266).

Although ionic Mn^{++} will activate a large number of different kinds of enzymes *in vitro*, its essential biochemical function is still in doubt (32). Most of the Mn in the body exists in readily dissociable complexes (33), and would presumably be available for combination with chelating agents. There are currently no well-defined stable manganese protein complexes which have been studied in relation to chelating agents. This survey will therefore be concerned with Fe, Cu, Zn and Mo proteins.

The most satisfactory proof for the removal of a metal from a metalloprotein by a metal-binding agent is obviously an analysis before and after treatment. Presumptive evidence of removal is obtained when the typical color of the metal-chelator-complex is obtained in dialyzable or extractable form upon the addition of the chelator to the pure metalloprotein; if the color remains associated with the nondialyzable protein, this is presumptive evidence of combination *in situ* without removal of the metal from the protein. Similarly, the inhibition of enzymatic activity by a chelator without loss of metal from the enzyme indicates *in situ* chelation. Metal chelators complex with some enzymes *in situ* to give the typical nondialyzable color without inhibiting the enzymatic activity—either because the free coordination bonds of the metal in the enzyme are not required for enzymatic activity, or because the chelator is replaced from the metal by the substrate. Based upon these premises, the known information about the well established Fe (and Mo), Cu and Zn proteins is summarized in Tables 1, 2 and 3, respectively.

IRON PROTEINS

About ¾ of the total 3–5 gm of Fe in the human body is present as hemoglobin; 16 per cent is in a storage form as ferritin, 3 per cent in myoglobin, and only small amounts in the transport form, transferrin, or in the iron-containing enzymes (49). Only 133 mg are estimated to be in a labile Fe pool rapidly available for hemoglobin synthesis (45). Once absorbed, Fe is not excreted, and it leaves the body in significant amounts only by bleeding.

Transferrin (Siderophilin, Metal-Binding β_1 Pseudoglobulin) (50, 148, 150)

Transferrin comprises 2–4 per cent of the plasma proteins (31, 112), contains all the 0.1–0.12 mg per cent of plasma Fe, and could combine with 3 times as much Fe as is normally present (221). Free Fe in excess of the saturation level of transferrin rapidly leaves the blood stream and produces toxic effects (148, 221). Transferrin binds Fe so tightly at physiological pH (150) that it will inhibit the growth of *Shigella dysenteriae* by making the medium deficient in Fe (225). Release of Fe from the plasma transferrin to the cells would seem to be possible only by reduction of the iron to Fe^{++} (149, 263). The absolute level of Fe in the serum is not directly related to the rate of Fe turnover (108, 221), Fe absorption or hemoglobin level (45).

The colorless Fe free transferrin from human plasma is a β_1 pseudoglobulin, free of SH groups, and has a molecular weight of about 90,000 (138, 152). At pH 7.5 it reacts with 2 atoms of Fe and 2 HCO_3^-, with replacement of 4 protons, to give a yellow color with Fe^{++} and salmon pink color with Fe^{+3}. The Fe^{++} form is readily oxidized to Fe^{+3} in air (226); and the salmon pink color of the naturally occurring Fe^{+3}- complex is discharged when the Fe is reduced by $Na_2S_2O_4$ (138) or when the Fe is dialyzed away below pH 5 (152, 253). The color of human (253) or pig serum transferrin (151) is similar to the color of the Fe^{+3}-complex formed with aspergillic acid or hydroxylamine (58). Transferrin Fe has ionic bonding characteristics (54, 253), and the Fe^{+3}-bicarbonate complex appears to be tied to the protein through combination with phenolic hydroxyl groups (138, 151). Transferrin also reacts with Cu, but the Cu of the green complex is easily replaced by Fe at physiological pH (138).

There is a measurable dissociation of Fe from transferrin at pH 7 when the Fe is reduced in the presence of OP (149). However, this dissociation must be relatively small, since serum Fe does not react directly or rapidly with BP or OP in the presence of reducing agents (148). In determining the iron-binding capacity of serum, the Fe added in excess of that required to saturate the transferrin can be removed by reacting it with OP in the presence of $Na_2S_2O_4$; this procedure does not remove much of the transferrin-bound Fe. The only way in which

TABLE 1. *Effect of Iron-Binding Agents on Naturally Occurring Iron and Molybdenum Proteins*

Enzyme or Protein	Source	Amounts of Fe, Flavin, and Mo per Mole	Agent Used*	Inhibition of Enzyme Activity	Removal of Fe or Mo by Agent			Combination With Fe or Mo *in situ*	Inhibition by CN
					Rapidly	On incubation or dialysis	On reduction		
Transferrin	Human plasma	2 Fe^{+3} 2 HCO^-	2, 4		No	No ?	Slow	No	?
Conalbumin	Egg white	2 Fe^{+3} 2HCO_3^-	4		No	No ?	Slow	No	No ?
Ferritin									
99 Fe^{+3} 1 Fe^{++}	Liver or spleen	$(Fe^{+3}OOH)_8$ $Fe^{+3}OPO_3H_2$	1, 2, 3, 4	Yes	No Yes	No ?	Yes	?	No ? Yes
Heme	Hemoglobin	Fe^{++}, Fe^{+3}	1, 2, 4	No	No	No	No	No	Yes
Homogentisicase	Beef liver	? Fe^{++}	1, 2, 3, 4, 5	Yes	?	?		?	Yes ?
Succinic dehydrogenase	Beef heart	2 Fe^{+3}·Flavin 4 Fe^{+3}·Flavin	1, 2, 3, 4 1, 2, 4	No On incub.	No No	No 2/4	? ?	Yes Yes	No No
DPNH-cyto c reductase	Pig heart	4 Fe^{+3}·Flavin	1, 4	On incub.	No	Yes	?	?	No
Lactic dehydrogenase	Bakers yeast	Porphyrin·1FMN†	1, 4	No	No	?	No ?	?	No
Xanthine oxidase	Milk	8Fe^{++}·2FAD‡·Mo	1, 2, 3, 4	No	No	No		Yes	Yes
Aldehyde oxidase	Pig liver	Porphyrin·2FAD‡·Mo	1, 3, 4	Yes/No ?	?	?	?	?	Yes
Nitrate reductase	Neurospora	?FAD‡·Mo	2, 3, 5 1, 4	No Yes	?	?	?	?	Yes

* *1*, 8 HQ; *2*, BP; *3*, EDTA; *4*, OP; *5*, DDC (see footnote, p. 158). † FMN, Flavin mononucleotide. ‡ FAD, Flavin adenine dinucleotide.

the Fe can be removed easily and completely from transferrin is at a pH less than 6, in the presence of an iron-binding agent such as OP, and with simultaneous reduction of the Fe by $Na_2S_2O_4$ (148) or cysteine (54).

Conalbumin

Conalbumin is an iron-binding protein (5) similar to transferrin (226); it constitutes 12–15 per cent of the proteins in egg white, is composed of a single peptide chain (65) with a molecular weight of about 76,000 (260), and is free of SH groups (288). The colorless metal-free protein reacts with 2 Fe^{+3} and 2 HCO_3^- ions to displace 6 protons; each atom of Fe, therefore, appears to be bound to 3 tyrosine phenolic groups in the protein; two additional bonds of the Fe are attached to HCO_3^-, and the remaining sixth bond holds an unknown ligand (288). Any disruption of the molecule which alters its architecture causes a loss of its iron-binding properties (64).

The ferric complex with conalbumin has the same absorption spectrum as transferrin (63), and the spectrum is unaltered by CN^- (288). Iron dissociates between pH 5–6 with loss of color, and the metal can be removed completely by complexing it with citrate at pH 4.7 (287). Excess free Fe can be removed at physiological pH with OP and $Na_2S_2O_4$ without simultaneously removing the conalbumin-bound Fe; however, there is a slow release of Fe from conalbumin by prolonged exposure to OP in the presence of reducing agents (64).

Ferritin (76, 77, 196)

Ferritin and hemosiderin are storage forms of Fe in liver and spleen (162, 259). Hemosiderin granules are insoluble crystals or aggregates of the soluble ferritin (224). They have a higher Fe content, and, on aging, hemosiderin becomes a very insoluble mineral. Both hemosiderin and ferritin in the liver are involved equally in the uptake of dietary Fe, and Fe is mobilized from both (90, 238). When injected intravenously, ferritin has vasodepressive and antidiuretic effects (230).

Apoferritin is a colorless globulin of molecular weight 460,000 which is synthesized in response to a stimulus created by the Fe itself. It incorporates within the apoferritin lattice (56) micelles of ferric hydroxidephosphate, $(FeOOH)_8$ $(FeOPO_3H_2)$, up to a total concentration of about 23 per cent Fe. The phosphate can be removed selectively to some extent (78), and a phosphorus-free, synthetic ferritin has been prepared by reacting apoferritin with Fe^{++} in a HCO_3^- buffer (21). The Fe must be ferrous in order to react with the apoferritin, but is then oxidized to ferric by air (161). The Fe in ferritin is unusual for biological compounds since it has only 3 unpaired electrons—hence only 4 coordination bonds; the latter are attached to 4 ligands in a square plane (14, 197). It does not react with CN^- on the basis of an unaltered absorption spectrum (197).

The reduction of ferritin Fe at pH 4.6 with $Na_2S_2O_4$ or at pH 7.4 with ascorbic acid, cysteine or GSH allows the Fe to be removed by dialysis, by chelating agents or by precipitation of the undenatured apoferritin (21, 79, 188, 191). Presumably, the Fe is released from ferritin physiologically by reduction to Fe^{++}; and liver homogenates are capable of effecting this reduction by virtue of their flavoprotein enzymes, especially diaphorase (254) and xanthine oxidase (189, 254).

A small proportion of the total Fe in ferritin is nor-

mally Fe^{++} and reacts directly with BP at neutral pH (188); this Fe^{++} is stabilized by associated SH groups, and is essential for the vasodepressive and antidiuretic effects of ferritin (230). By virtue of this Fe^{++}, ferritin can catalyze the oxidation of sulfide to thiosulfate—a reaction which is inhibited by CN^-, BP, 8HQ or by dialysis *vs* EDTA (13). Ferritin also oxidizes adrenalin to adrenochrome in the presence of H_2O_2 at acid pH (190), or in the presence of air at pH 7.4 (81). The latter reaction is inhibited by OP and BP, but is markedly stimulated by EDTA. The EDTA removes about 1.4 per cent of the total iron from ferritin at pH 7.4 to form an iron chelate which is a better catalyst for the oxidation of adrenalin than is the original ferritin.

Iron Porphyrins (154, 252, 258)

In all of the iron-porphyrins, 4 of the 6 coordination bonds of the Fe are tied to the pyrrole rings in a flat square structure; one of the remaining bonds projects above, and the other below the plane of the ring. In hemoglobin, myoglobin (198), catalase, and peroxidase one coordination linkage binds the Fe of the Fe porphyrin to the protein, and the remaining bond is free to unite with oxygen (in hemoglobin and myoglobin) or hydrogen peroxide (in catalase and peroxidase). This bond is also free to combine with other ligands in competition with the O_2 and H_2O_2; and, in the absence of such substrates, it is normally bound to water (hemoglobin) or a hydroxyl group (peroxidase and catalase). In cyto c both of the remaining coordination bonds of the Fe porphyrin are tied to histidine residues of the protein; and, in addition, the two vinyl groups of the porphyrin are firmly attached to SH groups in cysteine residues of the protein. This structure is therefore very stable, has no free coordination bonds by which it could unite with O_2, H_2O_2 or other ligands, and is limited to electron transfers. For the same reason cyto c cannot readily react with or be inhibited by such iron-binding agents as CO, S^-, N_3^-, CN^-, or hydroxylamine at physiological pH. On prolonged incubation, one of the coordination bonds of cyto c can be disrupted and combined with CN^-.

Carbon monoxide reacts with and inhibits a Fe^{++}-porphyrin-protein derivative possessing a free coordination bond. It therefore reacts with the Fe^{++} of hemoglobin or myoglobin to displace oxygen from the sixth coordination bond, but it does not react with catalase or peroxidase because the Fe^{+3} normally present in these enzymes is not reduced during enzymatic activity. Cyanide reacts primarily with and inhibits a Fe^{+3}-porphyrin-protein derivative possessing a free coordination bond. It therefore does not form a stable derivative with hemoglobin, but it does inhibit catalase and all the peroxidases from horse-radish, milk, adrenals, and leucocytes. The inhibition of horse-radish peroxidase by CN^- is competitive with H_2O_2, since the latter can replace the CN^- from the enzyme. Catalase and the peroxidases are also inhibited by other iron-binding agents such as S^-, N_3^-, or hydroxylamine, and the free coordination bond of hemoglobin Fe will also react with S^- or N_3^-.

Since cytochromes a and b undergo a Fe^{++}-Fe^{+3} valence change in the course of their enzymatic functioning, and, since they do not combine with either CO or CN^-, the Fe porphyrin is presumably tied to these proteins in the same way as in cyto c. Cytochrome oxidase also undergoes a Fe^{++}-Fe^{+3} valence change, and is inhibited by CN^-, CO, S^-, N_3^-, or hydroxylamine It therefore appears to have only one of the coordination bonds of Fe tied to the protein, while the other is free to combine with oxygen or other ligands.

The same protoporphyrin IX structure is present in catalase, peroxidase, cytochrome c and hemoglobin. When separated from the protein, the Fe protoporphyrin (heme) has 2 free coordination bonds which are normally bound to water but which will combine with 2 pyridine molecules to form a hemochromogen. The ferrous form of heme combines with only one CO, and the remaining water molecule can further be replaced by pyridine. The ferric form of heme (hematin) is normally bound to 1 molecule of water and 1 hydroxyl group, and the water can again be replaced by pyridine. Both the Fe^{++} and Fe^{+3} forms of heme react with 2 molecules of cyanide; CN^- displaces water and hydroxyl groups from both structures. The Fe in heme does not combine with quinoline, BP or, OP, and is not removed by such agents. Lemberg and Legge (154) state that "OP does not react with normal heme compounds in which the iron is inaccessible to (its) chelating influence." Since this reaction does not take place with free heme, it would not be expected to take place with the heme-proteins at physiological pH.

Oxygen Transferases (*Homogentisicase*)

Several enzymes which utilize molecular oxygen directly in the splitting of a benzene ring contain Fe^{++} as the prosthetic group (181). The Fe may be lost from the enzyme during purification, and such preparations then exhibit an absolute requirement for added Fe^{++}. Such enzymes include bacterial pyrocatechase and protocatechuic acid oxidase, as well as liver homogentisicase and 3-hydroxyanthranilate oxidase. The latter is inhibited by BP, OP, or EDTA, and these inhibitors are reversed by Fe^{++}; CN^- has little effect.

Homogentisicase from beef liver utilizes molecular oxygen (35) in the splitting of homogentisic acid to maleylacetoacetic acid (137). Exposure of the enzyme to pH 5 or dialysis at this pH inactivates the enzyme by removal of the Fe (137, 262), and such Fe-free or Fe-poor preparations are specifically reactivated by Fe^{++} but not Fe^{+3} (34, 137, 229, 251, 262). Glutathione and ascorbic acid help to activate the enzyme as well as protect against storage inactivation (34, 137, 229, 262). The inhibition by SH reagents is prevented by substrate but not Fe^{++} (262) and is reversed by GSH (137) or a mixture of GSH plus Fe^{+3} (34); Fe is not released by SH reagents (262).

The Fe appears to be bound to phenolic groups in the protein (262); it does not undergo a valence change in the course of enzymatic action, but it becomes "mobilized" and capable of exchanging with environmental Fe under these conditions (262). The natural iron-containing enzyme can be dialyzed at pH 7 without loss of Fe or

activity; it is strongly inhibited (34, 137, 229, 251, 262) by BP, OP, EDTA, 8HQ, DDC, hydroxylamine, and semicarbazide; the inhibition by carbonyl reagents as well as EDTA is reversed by Fe^{++} (229). Cyanide and N_3^- have been reported to be inhibitors (34, 137, 229), but others have found no effect (251, 262); CO does not inhibit (137).

METALLOFLAVOPROTEINS (169)

Succinic Dehydrogenase (243)

Succinic dehydrogenase from beef heart is an amber colored iron flavoprotein whose absorption spectrum reflects the amount of Fe present (241, 243). The freshly prepared enzyme contains 4 Fe^{+3} (183) and one flavin nucleotide per 200,000 molecular weight, while an aged preparation contains only half as much Fe and half the activity (241, 243). The Fe, but not the flavin, is liberated by boiling or cold acid (242). At least 2 of the 4 atoms of Fe present are not involved in electron transfers (183, 184). The spontaneous loss of activity on storage or handling is not prevented by EDTA or GSH (244), but is partially prevented by chelation of the Fe^{+3} with pyrocatechol disulfonate (184). Sulfhydryl reagents rapidly inactivate the enzyme, alter the spectrum to one which is more characteristic of simple flavoproteins, expose the Fe for rapid reaction with all chelators, and allow at least 2 of the 4 atoms of Fe to be removed from the enzyme by dialysis (184). The SH-inactivation can be reversed by GSH or CN^- (244).

The enzyme is not affected by prolonged incubation with CN^- (243) or BAL (244). The inhibition by transferrin can be prevented or partly reversed by Fe^{++} (118). The enzyme containing 2 Fe^{+3} is not inhibited by 8HQ, BP, or EDTA, and the Fe is not removed from the enzyme by prolonged incubation with such chelators (118, 243). OP forms a nondialyzable red complex with the enzymatic Fe *in situ*, but this inhibits the activity only slightly (118, 243). The enzyme containing 4 Fe^{+3} is more readily inhibited by 8HQ, OP or BP, especially with prolonged treatment, and some of the Fe becomes dialyzable on long incubation with OP (243). It would appear that all 4 atoms of Fe are required for maximal activity but 2 can be removed by SH reagents or prolonged treatment with chelators with a concomitant loss of 50 per cent of the original activity. However, the remaining 2 atoms of Fe will react with chelators *in situ*, but there is no further removal of Fe and no effect of the *in situ* chelation on enzymatic activity.

Succinic dehydrogenase from baker's yeast is very similar in chemical composition and enzymatic activities to the enzyme prepared from beef heart (245). It contains Fe and flavin in a 4:1 ratio in a molecule weighing 200,000. It is inhibited by SH reagents, but is not affected by CN^- or EDTA, and is only slightly inhibited by 8HQ. The partial inhibition by OP is competitive with the phenazine dye used in measuring the activity.

DPNH-Cyto c Reductase

The enzyme prepared from pig heart contains 4 atoms of Fe and one molecule of an unidentified flavin nucleotide (172) per molecule weighing 80,000 (168). Sulfhydryl reagents inactivate the enzyme and alter its spectrum irreversibly (168). The Fe can be removed from the enzyme by acidification or by dialysis *vs* 8HQ, and there is reported to be a concomitant loss of activity toward the reduction of cyto c but little or no loss of activity with dyes as electron acceptors (168). The partial inhibition produced by incubation with OP can be increased by preincubation of the enzyme with reduced diphosphopyridine nucleotide (DPNH) and can be restored by Fe^{+3}. The enzyme is not inhibited by CN^-, BAL, EDTA or antimycin A (282).

There is no indication from the absorption spectrum of the presence of Fe or any other metal in pig liver reduced triphosphopyridine nucleotide (TPNH)-cyto c reductase (104).

Baker's Yeast Lactic Dehydrogenase (Cytochrome b_2)

This enzyme contains one protohematin, one flavin mononucleotide (FMN) and an unidentified desoxyribose polynucleotide in a minimum molecular weight of 80,000 (8, 9). Using L-lactic acid as a specific substrate, the enzyme reduces cyto c, ferricyanide and MB; oxygen is reduced slowly. The reaction proceeds by an initial reduction of the enzymatic flavin, followed by a reduction of the enzymatic heme (9). It is very unstable, and the FMN tends to dissociate irreversibly on dialysis or storage (8, 9); EDTA provides some protection against this type of inactivation and thereby enhances the activity of the enzyme (23). The enzyme is inactivated by SH-reagents (8, 23), but is not inhibited by CO (11), CN^- or N_3^- (23), antimycin A (8), OP or 8HQ (24). The reported presence of 8 atoms of non-heme Fe in the molecule (24) has not been substantiated (9); this "impurity" Fe complexes with OP *in situ* to form a nondialyzable complex which has no effect on the enzymatic activity and does not remove the Fe (24).

Xanthine Oxidase (46, 293)

Three different xanthine oxidizing enzymes have been described. All three have been reported to contain one atom of Mo per molecule; in addition, they contain Fe and FAD in the ratios of 8:2; 4:1, and 8:1 for the milk, rat liver and chicken liver enzymes, respectively. Xanthine oxidase from rat liver is very similar to milk XO, except that the oxidase activity of the former can be inhibited relatively specifically by chalcones or antabuse. Both of these enzymes react rapidly with MB or O_2, but slowly with cyto c. The chicken liver xanthine dehydrogenase has a negligible reaction with oxygen but a good reaction with cyto c. All three enzymes reduce nitrate slowly; the optimum pH for nitrate reduction is approximately 5, while the optimum pH for the reaction with O_2 or MB is approximately 8 (292). All three enzymes oxi-

dize DPNH slowly and at a different enzymatic site from the purines or aldehydes. The purine dehydrogenase activity of all three xanthine enzymes is blocked by CN^-, 6-pteridyl aldehyde or hydroxylamine. The reaction of both oxidases with oxygen is blocked by semicarbazide, but it has no effect on the dehydrogenation of the substrate and the reduction of MB. Milk XO is inhibited by SH-reagents, but neither the milk nor rat liver enzyme is affected by BAL.

Milk XO

The Mo is present as an anion (292), presumably as molybdate or a molybdate complex, with Mo^{+6} having a coordination number of 8. The Fe^{++} seems to remain in the reduced form throughout the catalytic functioning of the enzyme (26). When substrate is added, the flavin adenine dinucleotide (FAD) is reduced rapidly (89) through a semiquinone, and the Mo is reduced from Mo^{+6} to Mo^{+5} (26). The way in which these three components are related to each other and are attached to the protein in the intact enzyme is completely unknown. On dialysis after aging, the Mo is removed more readily than the FAD; on dialysis *vs* 0.2N NH_4OH there is a parallel loss of Mo and FAD; in both cases the Fe is held tightly by the protein, and the enzymatic activity parallels the Mo content of the enzyme (292). Similarly, any removal of Fe by dialysis at pH 4.6 *vs* BP is accompanied by a corresponding loss of activity (66, 67). While each of these components may have an independent attachment to the protein, they appear to be interrelated in such a way that removal of any one completely disrupts the enzymatically active portion of the molecule.

Since flavin chelates tightly with Fe^{++}, an Fe^{++}-flavin complex might be anticipated in the enzyme. This concept is supported indirectly by the absorption spectra and the reaction with oxygen exhibited by different xanthine oxidizing enzymes. The absorption spectrum becomes more atypical of flavoproteins as the Fe-FAD ratio increases from 4:1 in the milk enzyme to 8:1 in the chicken liver enzyme, and in both cases the amount of color exhibited by each enzyme far exceeds that which would be contributed by these two components as independent entities. With an Fe-FAD ratio of 8:1, the FAD is no longer autooxidizable in air; with an Fe-FAD ratio of 4:1 in the milk enzyme, the flavin is autooxidizable, but the reaction with oxygen can be blocked by binding the Fe with semicarbazide, and can be restored by adding other Fe-binding agents, such as 8HQ, OP or EDTA (291).

The presence of 2 moles of FAD in milk XO provides 2 binding sites for the substrate (97) and the CN^--inactivated enzyme binds 1 mole of substrate for each FAD (66). Cyanide could react theoretically with either the Fe or the Mo, but the inactivation produced by combination with 2 moles of CN^- seems to be due to a reaction with the Mo. Since the reaction with CN^- is slow, some relatively stable linkage must be disrupted, but the enzyme can be reactivated partially by dialyzing the CN^- away *vs* $Na_2S_2O_4$ (66). There is no obvious reason why reducing agents would protect the enzymatic Fe^{++} from CN^-, while a reduction of Mo^{+6} to Mo^{+5} might prevent or disrupt the CN^- reaction; similarly, a reduction of Mo by the substrate and a complexing of the Mo with boric acid could account for the protection against CN^- inactivation which is provided by these substances. The spectrum of the enzyme is not altered by treatment with CN^- or CO, and this is compatible with the absence of spectral effects from the presence of Mo. All enzymatic activities are lost with CN^- inactivation, and the FAD can no longer be reduced by the substrate (291). Clearly FAD, Fe and molybdate function as a closely integrated unit in xanthine oxidase.

Metal-binding agents such as 8HQ, OP, BP, EDTA and N_3^- do not affect the activity of the enzyme with respect to substrate dehydrogenation and reaction of the reduced enzyme with MB or oxygen (48, 223). Some of these chelators actually increase the slow reactions of the reduced enzyme with cyto c (48) or nitrate (293), and these reactions are stimulated even more by the addition of Fe^{++} with a chelator such as 8HQ. The Fe is not removed from the enzyme by dialysis *vs* 8HQ (48), but the 8HQ presumably combines with the Fe or Mo *in situ* on the basis of its effect on cytochrome reduction. The typical color of this metal chelate with Fe has been seen in association with the nondialyzable enzyme, but the possible presence of extraneous Fe was not rigidly eliminated; 8HQ will also react with Mo^{+5} (206). Both Fe and Mo can be removed by dialysis *vs* 0.2 N HCl (223).

Aldehyde Oxidase

The role of Fe in the aldehyde oxidase of pig liver is not yet clear. The highly purified enzyme is free of nonporphyrin Fe but contains Fe porphyrin, Mo, and FAD in a 1:1:2 ratio (166); however, the hematin may not be an integral part of the enzyme (171). Cyanide or N_3^- alters the spectrum and immediately inhibits the reduction of cyto c by this enzyme; CN^- also inhibits dye reduction after incubation (171). The metal chelators 8HQ, OP and EDTA are reported also to have an immediate inhibiting effect on cyto c reduction without having any effect on dye or oxygen reduction even after incubation (171). All of the Mo can be removed from the enzyme by dialysis against 0.01 M NH_4OH; this has no effect on the spectrum, and the inactivation so produced can be restored by silico molybdate (75, 171).

Nitrate Reductase (Neurospora)

This catalyst for the reduction of nitrate by TPNH contains an easily removable FAD (202) and a more tightly bound Mo (134, 204). In its normal functioning, electrons are transferred from TPNH to the enzymatic FAD, thence to the molybdate portion to reduce Mo^{+6} to Mo^{+5} (206), and finally to the nitrate acceptor (205). SH-reagents inhibit the initial reduction of the FAD by the substrate, but have only a slight effect on the subsequent reduction of the nitrate; the SH-inhibition can be reversed by GSH or cysteine.

TABLE 2. *Effect of Diethyldithiocarbamate (DDC) and Cyanide (CN) on Copper-Containing Proteins*

Enzyme	Source	Color	Cu/mole	Removal of Cu by DDC			Combination With DDC *in situ*	Inhibition of Enzyme by DDC	Effect of CN	
				Rapidly	On incubation or dialysis	On reduction			Inhibition	Removal of Cu
Ceruloplasmin	Pig or human serum	Blue	8 Cu	No	No	Yes	Yes	Yes	Yes	Yes
Erythrocuprein	Ox RBC	Blue	2 Cu	No	No ?	?	?			?
	Human RBC	Pale blue-green	2 Cu	No	No ?	?	No ?			Yes
Cerebrocuprein I	Ox or human brain	Blue-green	2 Cu	No	No	?	?			?
Hepatocuprein	Ox or horse liver	Colorless	2 Cu	No	No ?	?	No ?			?
		Blue-green	2 Cu^{++}	?	?	Yes ?	?			Yes
Uricase	Pig liver	Colorless	1 Cu^{++}	No	No	?	Yes	No	Yes	No
DOPA oxidase	Mouse melanoma	?	?	?	?	?	Yes ?	Yes	Yes	Yes
Hemocyanin	Molluscs and arthropods	Blue	1 Cu^{+} 1 Cu^{++}	No	No	?	?		Yes	Yes
Tyrosinase	Potato, mushroom	Colorless	4 Cu	?	?	?	Yes ?	Yes	Yes	Yes
Laccase	Lacquer tree	Blue	4 Cu	Yes ?			?	Yes	Yes	Yes
Ascorbic oxidase	Squash	Blue-green	6 Cu	No	No	?	Yes	Yes	Yes	Yes

Cyanide or N_3^- produces an immediate inhibition without loss of Mo from the enzyme (134, 204). Incubation with CN^- removes the Mo, and the Mo-free enzyme is inactive with respect to nitrate reduction, but is still able to accept electrons from TPNH and pass them on to dyes or external flavin acceptors. The nitrate reductase activity is restored by the addition of sodium molybdate (205). Hence, Mo in this enzyme is involved only in the secondary transfer of electrons from the flavin portion of the enzyme to the ultimate nitrate acceptor. It is also possible for certain dyes to reduce the enzymatic Mo and then, secondarily, the nitrate, and under these conditions the flavin component of the enzyme is not required (205). The enzyme is inhibited only slightly by EDTA, DDC or BP but is reduced to 50 per cent of its original activity by OP or 8HQ (202). If the enzyme is free of metals other than Mo, these inhibitors must reflect a reaction with the molybdate portion of the enzyme.

COPPER PROTEINS (193)

The adult human body contains 100–150 mg of Cu, half of which is in the muscles; the liver contains 18 mg and has the highest concentration per unit weight (295). Blood contains approximately 0.1 mg per cent Cu distributed equally between the plasma ceruloplasmin and the RBC erythrocuprein (295). A Cu deficiency may lead secondarily to a typical iron-deficiency anemia (295).

Hepatolenticular degeneration (Wilson's disease) (30) is characterized by a genetic defect in the synthesis of ceruloplasmin, a low plasma and fecal Cu, an increased urinary excretion of Cu and a net positive Cu balance. Ceruloplasmin is reduced markedly, and a much higher percentage of the plasma Cu is bound loosely to albumin in a form which will react directly with DDC. Copper accumulates in large amounts in the liver and in the affected portions of the brain. The administration of ceruloplasmin intravenously to restore normal levels of this compound in the plasma does not appear to correct the disorder (20).

Ceruloplasmin

The role of ceruloplasmin in the absorption and transport of Cu (86, 237) is still uncertain. Although 95 per cent of the normal plasma Cu is present as ceruloplasmin, the latter does not seem to act as a mucosal block; newly absorbed Cu is loosely bound to serum albumin rather than ceruloplasmin (15) and is normally removed rapidly from the plasma and subsequently converted to ceruloplasmin. In Wilson's disease there is no secondary conversion to ceruloplasmin; the Cu remains in a "direct-reacting" form for a longer period of time, and is, therefore, more available for deposition in the brain or excretion in the urine (15). Ceruloplasmin has a half life of 0.92 day; both the Cu and the protein portions are handled as a single unit, and there is little exchange with dietary Cu (74). No plasma protein other than ceruloplasmin can bind Cu in a way which prevents a direct reaction with DDC at neutral pH.

Ceruloplasmin prepared from human or pig serum is a blue α_1 globulin containing 8 atoms of Cu per molecule weighing 151,000 (102). Four of the Cu atoms seem to be bound differently, since all of the color but only 50 per cent of the Cu is removed by digestion with chymotrypsin (38), and only 4 of the 8 Cu atoms exchange with environmental Cu in the presence of ascorbic acid (227). Ceruloplasmin Cu does not react directly with most ion exchange resins (227) or with DDC (101) but can be removed partially by incubation with penicillamine (286). Dialysis below pH 5 removes the Cu (103) and the enzymatic activity of ceruloplasmin; the latter can be restored by Cu^{++} (4). Cyanide bleaches the color (228), and dialysis *vs* CN^- at pH 7 causes a loss of Cu, color and enzymatic activity—none of which is restored by added Cu (103). The divalent Cu^{++} in the blue ceruloplasmin

can be reduced reversibly to a colorless Cu^+ form by $Na_2S_2O_4$ or ascorbic acid, and reoxidized by air (102, 227). When the Cu in ceruloplasmin is reduced, it is possible to remove it with DDC (235).

Ceruloplasmin has weak enzymatic activity which is characteristic of the plant laccases. As such it preferentially oxidizes *p*-phenylenediamine, but it can also oxidize ascorbic acid and some *o*- and *p*-dihydroxyphenols without the production of H_2O_2; monophenols and monoamines are not oxidized (103). DOPA (103), adrenaline (39, 103, 153), and serotonin (39, 218) have been listed as substrates for ceruloplasmin, but also questioned (73). The oxidation of *p*-phenylenediamine or dimethyl-*p*-phenylenediamine is inhibited by CN^-, N_3^-, or DDC (4, 39, 103), hydroxylamine or semicarbazide (1), but not by CO (103), or EDTA (27).

Erythrocuprein

A blue crystalline Cu protein was originally isolated from the erythrocytes of ox blood (176) and a similar but pale blue-green glycoprotein has been obtained more recently from human RBC (133, 178). Erythrocytes take up Cu from plasma rapidly (29), and practically all of the 0.1 mg per cent of Cu present in RBC is in erythrocuprein (178). Both the human and bovine erythrocuprein contain 2 Cu per molecule (0.32–0.36 per cent Cu; mol. wt. = 33–35,000). Neither reacts directly with DDC, but the Cu can be removed at least partially from the human protein by dialysis *vs* CN^- at pH 7.5–8 (178). Neither has any recognized enzymatic activity. It is possible that these two erythrocupreins represent the Cu^{++} (blue) and Cu^+ (colorless) forms of the same molecule, since the blue protein from ox erythrocytes can be bleached irreversibly by $Na_2S_2O_4$; however some Cu^{++} proteins are colorless.

Cerebrocuprein I

Essentially the same blue-green Cu protein has been isolated from bovine (216) and human (215) brain. It contains 2 Cu per molecule (0.3 per cent Cu; mol. wt. = 30–40,000). The Cu is dialyzable only below pH 4.5; it does not react directly on standing with, nor is it removed by, treatment with DDC at slightly alkaline pH (214, 215). The protein as isolated has no enzymatic activity toward *p*-phenylenediamine or polyphenols (216). The Cu which accumulates in the brain in Wilson's disease is nondialyzable, and $\frac{2}{3}$ is present in the crude cerebrocuprein fraction (217); however, all of the Cu is not present as cerebrocuprein I, since some will react directly with DDC and will dialyze at pH 4.5 (214, 217).

Hepatocuprein

A colorless Cu protein was isolated from ox liver (176), and a somewhat different blue-green protein has been isolated from horse liver (199). They both contain 0.3–0.4 per cent Cu in a molecular weight of 30–40,000 (2 Cu/mole). Neither has any recognized phenolase or ascorbic acid oxidase activity. The blue protein can be bleached with $Na_2S_2O_4$, and the color restored by dialysis against Cu acetate (199). Similarly, the Cu can be dissociated from the protein by dialysis *vs* CN^- at pH 7.5 or by precipitating the protein with ammonium sulfate at pH 5 (199); in both cases the color and Cu can be restored by reacting the apoprotein with Cu acetate. The Cu in the ox protein does not react directly with DDC.

Uricase

Uricase oxidizes uric acid to an unstable intermediate which decomposes to allantoin and CO_2; molecular oxygen (but not dyes) acts as the electron acceptor, and H_2O_2 is produced (41, 123). The one atom of Cu per mole of colorless hog liver uricase (mol. wt. 120,000) is not even dissociated by 5% trichloroacetic acid (167). Two of the 4 coordination bonds of the Cu appear to be attached to the protein; the other two bonds normally are attached to one hydroxyl group and one water molecule but will combine with the substrate or other ligands in the medium (12). Cyanide combines with the Cu in the enzyme by replacing the hydroxyl group (12); this inhibits the enzyme, but the inhibition is reversed by dialyzing away the CN^- (41, 100, 123, 170). Dialysis against CN^- does not remove the Cu from the enzyme (170). The enzyme also binds DDC or 8HQ *in situ* to give the typical colors of the Cu complexes in nondialyzable form. These agents do not inhibit the enzymatic activity (41, 100) because they are replaced competitively by the substrate (170). Substances which reduce the Cu as well as bind it, such as ascorbic acid or hydroxylamine, inhibit the enzymatic activity, while substances which are merely potential binders of cupric ions do not (170); the enzyme is protected from the ascorbate or hydroxylamine inhibition by the presence of the substrate or other chelators (170).

Other substances which do not inhibit uricase (41, 100, 123, 170) include EDTA, OP, BP, dithizone, 8-hydroxyquinoline sulfonic acid, sulfanilamide, semicarbazide, S^-, F^-, CNS^-, or SH reagents (107). Azide inhibits only slightly or not at all (100, 123, 170). Carbon monoxide does not inhibit in the presence or absence of the substrate (41, 123). Excess substrate (12) or added Cu^{++} are inhibitory (12, 100, 123).

Mammalian Tyrosinase (DOPA Oxidase)

The melanin pigments found in mammalian eyes (7), skin and hair are formed by a Cu enzyme which is similar to the plant tyrosinases. An active enzyme is found in the melanocytes of human black hair matrix but not in albino or gray hair bulbs; a potential tyrosinase activity is present in epidermal melanocytes but is not activated until the skin is exposed to ultraviolet radiation (143). Melanized granules from a melanoma contain an active tyrosinase (52), which has been solubilized only recently (28). The conversion of DOPA to melanin by skin slices *in vitro* (22, 59, 62) is inhibited by DDC (60, 143), CN^-, S^-, or N_3^- (158).

Mammalian tyrosinase from human (130), horse or fish (61) and mouse melanomas oxidizes DOPA to melanin; tyrosine is oxidized also to DOPA and melanin after a lag period which is shortened by the addition of small amounts of DOPA or other dihydroxyphenols (157). When dialyzed *vs* CN^- the Cu content of the mouse melanoma preparation is decreased, and most of the activity is lost; the activity can be restored completely by Cu^{++}, but the reconstituted enzyme now exhibits an absolute requirement for small amounts of DOPA in the oxidation of tyrosine (130, 157). The enzyme does not oxidize catechol or adrenalin (130).

Tyrosinase from mouse melanoma is inhibited by BAL or DDC (157), CN^- (82, 99), α-naphthylthiourea (7, 92), *p*-benzylhydroquinone (99), *p*-aminobenzoic acid, cysteine, thiourea, or α-benzoinoxime (164), but not by sulfanilamide (164). The inhibition by cysteine, thiourea, α-benzoinoxime (164), BAL or DDC (61, 157) is reversed by added Cu^{++}.

Hemocyanin (43)

The hemocyanins are oxygen-carrying pigments (222) in the hemolymph of certain molluscs and arthropods. They contain 0.15–0.26 per cent Cu and have various molecular weights (116, 147) because of aggregation of smaller units. The oxygen-free colorless molecule becomes blue when exposed to air, and this reversible oxygenation is accompanied by a partial oxidation of the Cu. In the absence of oxygen the Cu is present as Cu^+; upon oxygenation one-half of the Cu is oxidized to Cu^{++} (136). One atom of Cu^+ and one atom of Cu^{++} combine with each molecule of O_2 (136).

The hemocyanin Cu is tightly bound to the protein (57), presumably through histidine residues (163). It is not removed by an Amberlite resin, nor does it exchange with environmental Cu in the presence or absence of oxygen (115). The Cu does not react directly with DDC but will do so after treatment with 60 per cent alcohol or with alkali (297, 298). The Cu is removed easily by acid, and the blue color disappears below pH 4. Dialysis *vs* CN^- at pH 7.4 removes the Cu with a concomitant loss of color and oxygen-carrying capacity; the apoprotein can be restored with Cu^+ but not Cu^{++} (142, 163) to reform the reversible oxygen-carrying pigment. The blue color is also bleached by $Na_2S_2O_4$ (115) or $NaHSO_3$ (135). Its reaction with CO is questionable (136, 142).

Plant Oxidases (43, 44, 179, 240)

Three Cu-containing enzymes from plant sources, tyrosinase, laccase, and ascorbic acid oxidase utilize molecular oxygen [but not dyes or cytochromes (80)] as the electron acceptor and do not form H_2O_2. All tyrosinases from mammalian, plant or insect sources oxidize tyrosine to melanin in the presence of a small amount of *o*-dihydric phenol (156). Plant tyrosinases also oxidize a variety of *o*-dihydroxyphenols and many monophenols, secondarily, but do not oxidize *m*- or *p*-dihydric phenols or *p*-phenylenediamine (80). Blocking the amino or carboxyl group of tyrosine does not prevent its oxidation by tyrosinase (247) and the tyrosine residues in a protein can be oxidized. The laccases preferentially oxidize hydroquinone and *p*-phenylenediamine but will also oxidize other *o*- and *p*-dihydroxyphenols; tyrosine is not oxidized by laccase in the presence or absence of catechol (80). Both tyrosinase and laccase oxidize ascorbic acid only in the presence of a natural substrate such as catechol (80), and ascorbic acid oxidase does not oxidize phenols significantly (165, 256).

Tyrosinase (155)

Tyrosinases from potatoes and wild or cultivated mushrooms are essentially colorless or pale yellow proteins containing 0.2–0.3 per cent Cu (40, 119, 142), or 1 atom of Cu in a minimum molecular weight of 30–35,000 (132), or 4 atoms of Cu per 100,000 molecular weight (173). Kubowitz (142) reported that the enzymatic Cu^{++} is reduced to Cu^+ by the substrate and then is reoxidized by oxygen. However, Kertesz (131) has presented evidence that the enzymatic copper is Cu^+ and remains in the reduced form throughout its catalytic action. The same enzyme catalyzes the dehydrogenation of *o*-dihydroxyphenols to *o*-quinones, as well as the utilization of molecular oxygen (182) for the introduction of a new hydroxyl group into monophenols or *o*-quinones (203). The lag period in the oxidation of monophenols is shortened or eliminated by a small amount of an *o*-dihydroxyphenol (119) or ascorbic acid (16, 126, 140). Ascorbic acid does not affect the oxidation of catechol (110) but is oxidized secondarily by the *o*-quinone formed in the reaction. Mason (180) has suggested that the dihydroxyphenol substrate or ascorbic acid reduces the enzymatic Cu, that 2 atoms of such reduced Cu^+ combine with one molecule of oxygen, and that such a combination acts as an oxygen transferase for the introduction of a new hydroxyl group into a monophenol. Synthetic complexes made with one Cu^{++} plus two molecules of pyridine, BP, OP, etc. can also catalyze the oxidation of DOPA to melanin (111).

The Cu can be dialyzed away from tyrosinase in dilute acid (40), or it can be removed by treatment with CN^- at neutral pH and subsequent dialysis (142); the enzymatic activity of the apoenzyme can be restored completely by adding an excess of $CuSO_4$ (142). The apoenzyme can also be prepared by pretreatment of crude extract with CN^-, and it can then be reactivated with Cu^{++} after purification (127, 129). The insect protyrosinase acts like the potato or mushroom enzyme with respect to CN^- (6); the enzyme from tea loses its Cu and activity with CN^-, but the apoprotein cannot be reactivated by Cu^{++} (248); the tyrosinase from belladonna is resistant to CN^- but is inhibited by CO and DDC (113).

Tyrosinase is inhibited by Cu-binding agents or by substrate analogs, and the formation of melanin is prevented also by substances which react with the intermediates formed in the reaction. One molecule of CO reacts with 2 atoms of reduced enzyme Cu^+ in a light insensitive, reversible reaction, which inhibits the enzyme (119,

142). The inhibition (84, 142, 257, 289) by DDC, salicylaldoxime, ethyl xanthate, or BAL can be reversed by adding Cu salts (142, 257, 289). In addition to S^- and N_3^- (119, 142), a variety of thiourea, sulfanilamide, and other derivatives also inhibit by complexing with Cu (19, 51, 93, 139, 144, 187, 208) and these inhibitions may or may not be reversed by added Cu ions (51, 208). A variety of phenols such as 4-chlororesorcinol and 2,4-DOPA (93, 144, 146) are substrate analog inhibitors.

Laccase

Laccase from the latex of the lacquer tree has been purified to a Cu content of 0.24 per cent (121). It contains 4 atoms of Cu in a molecule weighing 120,000, undergoes a one-electron transfer reaction, and has an oxidation-reduction potential of +0.415 v at 25°C and pH 7 (201). Its blue color can be bleached by the addition of hydroquinone substrate and restored by oxygenation (201); this is similar to the blue laccase from *Polyporus versicolor* which contains some Cu^{++} per molecule that is reduced to Cu^+ by substrate and reoxidized by oxygen (174, 175). The color of laccase is bleached irreversibly by heat, acid, CN^-, or DDC (120, 121). The activity (and presumably the Cu) can be removed from the enzyme by dialysis *vs* CN^- under reducing conditions, and then restored by the addition of Cu^{++} (261). The enzyme is inhibited by CN^-, S^-, N_3^- and DDC, but not by CO (120). A laccase from mushrooms is inhibited also by CN^- but exhibits remarkable stability to acid (83).

Ascorbic Acid Oxidase

The ascorbic oxidase from squash (or cucumber) (249) is a blue-green protein containing 0.26 per cent Cu, or 6 atoms of Cu in a molecule weighing 146,000 (53, 165, 220). In the absence of oxygen or upon the addition of ascorbic acid, the color is bleached to yellow—either because of a change in valence of the Cu or because of a change in the oxygenation of the molecule similar to that undergone by hemocyanin (53). The Cu cannot be removed by an Amberlite resin and does not exchange with environmental Cu except when catalyzing the oxidation of ascorbic acid aerobically (114). Copper is not lost from the enzyme during such catalysis, nor is it lost as a result of the inactivation of the enzyme which occurs spontaneously while the enzyme is carrying out its catalytic function (114).

The enzyme is stable to dialysis at pH 5.8 (192) but is decolorized with loss of activity at pH 4 (53). Copper is not removed by dialysis *vs* EDTA (71) but is removed by dialysis *vs* CN^-; the apoprotein can be reactivated by the addition of excess $CuSO_4$ (195). The enzyme is inhibited (55, 70, 71, 186, 192, 239, 249, 250, 256) by pyridine-KCNS, CN^-, S^-, N_3^-, DDC, 8HQ, ethyl xanthate, thiourea, salicylaldoxime, and by excess Cu^{++}, but not by CO. DDC combines with the Cu of the enzyme to give a colored complex and inactivate the enzyme (250), but the DDC can be dialyzed away at pH 5.9 without removing the Cu and with a full restoration of activity (192, 250). The inhibition of the enzyme by 3,4-dichlorophenylserine is reversed by Cu (284). The SH-inhibition produced by phenylmercuric chloride can be reversed by thyroxine (72), cysteine or GSH (71).

ZINC PROTEINS (91, 95, 268, 269,)

Zinc exists only in a single valence state with a characteristic coordination number of 4 (88). Its major binding sites in proteins have been identified as SH and imidazole, although amino and possibly carboxyl groups might be involved secondarily. Nonessential Zn can be added to or removed from enzymes by procedures which do not eliminate the essential Zn. Zinc proteins are colorless and do not have any major spectral characteristics by which the presence of Zn can be recognized.

Blood Z.

Whole blood contains 0.7–0.8 mg per cent Zn, 85 per cent of which is present in the red cell as carbonic anhydrase (277). About 3 per cent is present in the leucocytes firmly bound to a protein whose enzymatic function is unknown (275). The remaining 12 per cent is bound to plasma proteins in a nondialyzable form (17). However, treatment of serum with DDC or alumina gel extracts about ⅔ of the 0.125 mg per cent of Zn in plasma, while ⅓ remains attached firmly to the protein (283). Both the loosely bound and the firmly bound Zn are associated to some extent with all the plasma proteins, but most of the

TABLE 3. *Effect of Zinc-Binding Agents on Zinc Proteins*

	Source	Zn/mole	Agents Studied*	Inhibition of Enzyme	Combination With Zn *in situ*	Removal of Zn		Inhibition by CN
						Rapidly	On incubation or dialysis	
Plasma Zn	Human	Var.	1			2/3		
Carbonic anhydrase	Bovine RBC	1	1, 2, 3, 4	No	?	No	No	Yes
Carboxypeptidase	Pancreas	1	2, 5, 6	Yes	Yes	No	Yes	Yes
Alcohol dehydrogenase	Liver	2	1, 2, 6	Yes	Yes	No	No	Yes ?
Alcohol dehydrogenase•	Yeast	4	2, 3, 5, 6, 7	Yes	Yes	No	?	Yes ?
Glutamic dehydrogenase	Liver	2–4	1, 2, 3, 4	Yes	Yes	No	?	No

* *1*, DDC; *2*, OP; *3*, 8HQ; *4*, EDTA; *5*, BP; *6*, 8-hydroxyquinoline-5-sulfonic acid; *7*, diphenylthiocarbazide (dithizone).

loosely bound Zn is attached to albumin, and smaller amounts are associated with the β_1 globulin fraction. Most of the tightly bound Zn is attached to the α_1 and α_2 globulins (296). Plasma proteins are capable of binding at least 1 mg per cent of Zn in a nondialyzable form, and this added Zn has the loosely bound characteristics.

Carbonic Anhydrase

Bovine carbonic anhydrase contains 0.21 per cent Zn or 1 atom of Zn per molecule weighing 31,000 (159) in a tightly bound, nondialyzable form, which does not exchange *in vitro* with environmental Zn^{++} (265). It is inhibited reversibly by such general metal inhibitors as CN^-, S^-, or N_3^- (122), and more specifically by the sulfonamide grouping in sulfanilamide (42, 177). It is inhibited also by BAL (289), an excess of Zn^{++}, or thiocyanate (106). It is not inhibited significantly by such Zn-complexing agents as DDC, OP, 8HQ, EDTA or anthranilic acid (125), even after long incubation (42), and the Zn is not removed by dialysis *vs* OP under certain conditions (159). Either these agents are not bound by the Zn as it exists in the enzyme, or the complex is so weak that it can be displaced by the substrate and, therefore, not register in the activity measurement. Below pH 4 the Zn is removed with complete destruction of the enzyme (122, 125); however, the Zn can be removed at pH 5 by careful treatment with OP and the activity of the apoenzyme can then be restored fully by the addition of Zn^{++} (160). The acid inactivation of carbonic anhydrase is partially reversible on neutralization; this reactivation is not influenced by Zn^{++} but is inhibited by dithizone, EDTA or 8HQ (124).

Pancreatic Carboxypeptidase

The one atom of Zn per molecule (34) of pancreatic carboxypeptidase (278) cannot be removed by dialysis at pH 7 unless a complexing agent such as OP is also present (279). As the enzyme is freed of Zn by dialysis at pH 4.2 or by dialysis *vs* OP at pH 7, there is a parallel loss of enzymatic activity. A stoichiometric amount of Zn can be recombined with the apoenzyme to give a full restoration of activity.

The enzyme is inhibited by the addition of such complexing agents as OP, BP and 8-hydroxyquinoline-5-sulfonic acid, but not by DDC or Diamox (278). The OP is known to combine *in situ* with the enzymatic Zn in a reversible combination, because the complex has a characteristic u.v. absorption spectrum (271). The enzyme can also be inhibited reversibly by CN^-, or S^-, but not by N_3^- (246).

Alcohol Dehydrogenases

Liver alcohol dehydrogenase contains 2 atoms of Zn per molecule weighing 83,000 (273), while the yeast enzyme contains 4 atoms of Zn per 150,000 molecular weight (272). One molecule of DPN is attached to each Zn atom. Zinc in excess of the above amounts can be removed from these enzymes by dialysis at pH 6, and the essential Zn can be removed from the protein by dialysis below pH 6; a loss of enzymatic activity parallels the loss of essential zinc, but, once removed, the activity cannot be restored by the simple addition of Zn^{++}. The essential Zn does not exchange with environmental Zn^{++} during the preparation of the enzyme (276).

Zinc complexing agents, such as OP or 8HQ, inhibit both enzymes. This inhibition is instantaneous, competitive with DPN, and is reversed by dialysis or by adding Zn salts. Both the liver and yeast enzymes rapidly bind one molecule of OP per atom of Zn through the two free coordination bonds of the latter (96, 270, 281, 294). The other two coordination bonds of each zinc atom apparently are bound to the protein. Combination of the OP with Zn does not remove the metal from the enzyme but competes with DPN for this site, and the complex is, therefore, inactive and dissociable. On longer standing of the yeast enzyme with an excess of OP, two molecules of the inhibitor become attached to each atom of Zn; this irreversibly inactivates the enzyme, but the mechanism is unknown. The liver enzyme is not disrupted in this way by prolonged treatment with OP. While neither enzyme is rapidly inactivated by CN^-, both the liver (117) and yeast (268) alcohol dehydrogenases can be inhibited.

Liver Glutamic Dehydrogenase

This enzyme contains 2 to 4 (av. 3.4) atoms of Zn per molecule weighing 1,000,000, (2, 212). Excess Zn inhibits, presumably by combining with free SH groups (207). The crystalline enzyme can be dissociated into 3 or 4 subunits by dilution (141) or by treating it with DPNH or Zn-binding agents such as OP; this dissociation reversibly inactivates the enzyme (68). Apparently, the aggregate is formed from the subunits through Zn-DPN groupings since this effect of OP can be prevented by DPN, and the latter promotes the association of the molecule (69).

Enzymatic activity is inhibited by a number of Zn-binding agents, such as S^-, N_3^-, DDC, 8HQ, EDTA, CO, etc. (2), but not by CN^- (47). The inhibition by OP is similar to that exhibited by yeast alcohol dehydrogenase. There is an instantaneous reversible inhibition when OP is present in a 1:1 ratio with the Zn. On incubation with an excess of OP, the inhibition can no longer be reversed by dilution, dialysis or the addition of Zn^{++} (3).

Other Possible Zinc Enzymes

One of the alkaline phosphatases in kidney is activated by Zn carried as an amino acid complex (85). The best preparations of this enzyme contain about 0.15 per cent Zn; both the Zn and enzymatic activity are lost when dialyzed *vs* EDTA (185). Metal-complexing agents such as BP and CN^- activate the enzyme in low concentration but tend to inhibit at higher concentrations (185). A similar enzyme in calf duodenum is inactivated by binding EDTA in a nondialyzable form and is reactivated by Zn-glycine (98). An alkaline phosphatase in human leuco-

cytes is inactivated also by EDTA and restored by Zn^{++} (264, 267).

Other possible Zn enzymes which are inhibited by metal-binding agents and reactivated by Zn^{++} include lactic dehydrogenases from muscle (212, 280), and yeast (37), and other DPN-requiring dehydrogenases (274).

Zinc-Protein Complexes

Zinc combines with many proteins by reacting first with free SH groups and then with imidazole groups. Over 30 atoms of Zn can be combined with the free SH groups in each molecule of yeast alcohol dehydrogenase. This excess or nonessential Zn can be removed by dialysis in the presence of EDTA but not in its absence (285). Since the enzyme requires free SH groups for activity, the addition of excess Zn inhibits and its removal reactivates the enzyme. Clearly, the added Zn is bound differently from the essential Zn and is more readily removed by chelating agents.

Zinc reacts in a 1:1 ratio with the uncharged imidazole residues of serum albumin to form a loose complex; free amino and carboxyl groups are not involved (87). This binding is competitive with hydrogen ions since the imidazole group can be titrated in the physiological pH range. Insulin is free of SH groups and binds variable amounts of Zn (0.15–0.52 per cent) in proportion to the Zn concentration (36). Small amounts of Zn (0.1–0.3 atoms of Zn per 6000 molecular weight unit of insulin) are bound irreversibly. Larger amounts cause a greater accumulation of Zn as well as an aggregation of the protein; however the Zn in excess of 0.3–0.4 atom/6000 weight unit can be dialyzed away. In crystalline Zn-insulin one atom of Zn is attached to two imidazole groups (255).

CONCLUSIONS

Most of the Fe, Cu, Zn and Mo present in biologically functioning metalloproteins cannot be removed by exposure to chelating agents at physiological pH. Only Fe^{++} seems to be removed easily from the iron proteins, and even the Fe^{++} in the heme proteins or xanthine oxidase is bound tightly. Only the small amount of Fe^{++} present in ferritin and in enzymes such as homogentisicase would seem to be readily available to chelating agents. Relatively little iron is actually removed from the body by chelating agents (236). None of the Cu proteins give up their essential Cu readily except to cyanide. Zinc forms simple complexes with proteins which can be disrupted easily by chelating agents, but the essential Zn in the Zn enzymes is bound stably.

Considering the stability of the EDTA complexes to decrease in the order: Fe^{+3}, Cu^{++}, Zn^{++}, Fe^{++}, Mn^{++}, Ca^{++}, Mg^{++}, the reason for the zincuria after EDTA administration (210, 211) is understandable. Fe^{+3} and Cu^{++} cannot be displaced readily from their natural binding sites. Most of the Zn attached to serum proteins or other similar Zn-protein complexes is bound loosely and susceptible to removal by a chelating agent. Some Fe^{++} and Mn^{++} might also be expected to be available, but they are bound less tightly by the EDTA than the Zn and are not available in more than trace amounts in plasma.

Inhibition of enzymes by chelators cannot be generalized. Some metalloenzymes do not react with any metal chelator and are not inhibited; others react with selected, but not all, binding agents. Some enzymes react with a variety of metal-binding agents but are not inhibited; others are inhibited instantly or only after incubation. For *in vivo* inhibitions to be obtained, the concentration of the chelator as well as its passage through cellular membranes must be considered. An enzyme will not be inhibited automatically by a metal chelator simply because it contains a metal. Even cyanide, the classical detector of metals in enzymes, does not inhibit all metalloenzymes.

STUDIES OF XANTHINE OXIDASES

Metal chelators usually are thought of as potential enzyme inhibitors, but in a few cases they activate and/or protect a metalloenzyme against storage deterioration or "reaction inactivation," i.e., a destruction of the enzyme while it is carrying out its normal catalytic function. Succinic dehydrogenase and xanthine oxidase are protected partially against storage deterioration by pyrocatechol disulfonate (184) and by salicylate (18) or a naturally occurring impurity (25), respectively. The lactic dehydrogenase from baker's yeast retains its FMN and activity better in the presence of EDTA (23). Tyrosinase (10, 109) and ascorbic acid oxidase undergo extensive reaction inactivation. Such inactivation is not due to the reaction products or the loss of total metal from the enzyme (114) but the metal-protein linkage is weakened under the conditions of reaction inactivation. The metal in ascorbic oxidase (114) or homogentisicase (262) will exchange with environmental ions only during the course of the enzymatic reaction, and 4 of the 8 Cu atoms of ceruloplasmin will exchange only after reduction by ascorbic acid (227). Ascorbic oxidase is protected partially against reaction inactivation by inert proteins and heme derivatives (219) or by Cu-complexing agents such as EDTA, CN^-, DDC, 8HQ, amino acids or SH compounds (71). Substances such as CN^-, DDC or 8HQ activate and protect the enzyme in low concentrations but inhibit at higher concentrations (70, 71); EDTA and proteins protect at any concentration.

Milk xanthine oxidase undergoes a similar reaction inactivation, although it is not apparent until the enzyme is highly purified. It is protected both aerobically and anaerobically by "inert" proteins and by metal-binding agents. The complexing of the enzymatic metal with a foreign agent stabilizes the enzyme so that it can carry out its normal catalytic function without disruption of the molecule.

Stabilization of Purified Xanthine Oxidase

Aerobic. Milk XO was purified (105) by adsorption on alumina gel C_γ, elution with 0.5 M phosphate buffer pH

7.5, followed by adsorption on calcium phosphate gel, washing with 0.1 M phosphate buffer pH 6.2, and elution with 0.5 M phosphate buffer pH 6.2 After such purification, the enzyme was very unstable in the usual Warburg determination (291); the activity fell off rapidly, and there was not a good linear relationship between the measured oxygen uptake and the amount of enzyme present. The enzyme could be stabilized with respect to both of these characteristics by the addition of 5 mg albumin, 1 mg 8HQ, 5 mg BP or 0.15 cc of 0.01 M methylene blue. Albumin and MB added together gave more activity than either one alone. Catalase prevented the rapid destruction of activity during the determination but did not allow as much oxygen uptake per minute as did the addition of albumin. Glutathione or cysteine (5 mg) not only sustained the activity well but increased the rate of oxygen uptake by 50 per cent and 80 per cent, respectively, above that obtained with albumin.

Aldehyde substrates produce a rapid reaction inactivation when oxidized aerobically even by crude milk XO. With *p*-hydroxybenzaldehyde substrate and the purified enzyme, GSH and cysteine increased the activity and gave partial but not good stabilization. Albumin, 8HQ and BP had no effect; as with the control flasks, there was no linear relationship between oxygen uptake and the amount of enzyme present, nor was the activity sustained beyond the first 10 minutes of the measurement. Hence, the reaction inactivation obtained with aldehydes is different from that obtained with hypoxanthine substrate.

Anaerobic. The purified enzyme also failed to react rapidly and completely with MB, unless a stabilizer were present. The anaerobic system was composed of enzyme, 0.15 cc of 0.66 M phosphate buffer pH 7.5, 0.25 mg hypoxanthine and 0.05 mg MB in a total volume of 2.1 cc. Figure 1 shows the assay curves obtained with the purified enzyme in the presence and absence of albumin. At all levels of enzyme concentration more activity was exhibited in the presence of albumin, and only in the presence of albumin was there a linear relationship between the enzyme concentration and the decolorization time. Since the assay curve in the absence of albumin did not pass through the origin, it is evident that the enzyme was inactivated during the course of the reaction; this destruction was most evident when the total amount of enzyme present was small. As the purified enzyme aged for several weeks in the cold, it lost activity and the albumin effect decreased (Fig. 1). The additional protein present in the test system arising from the inactivated enzyme appears to have stabilized the remaining active enzyme.

By using an appropriate concentration of enzyme the effect of albumin on the reduction of MB was quite pronounced. With 0.1 cc of enzyme (Fig. 1) the MB was decolorized in 3.5 minutes when 5 mg albumin was present, but it required over 2 hours in the absence of albumin. In the absence of stabilizer, about half of the MB appeared to be reduced in 10–15 minutes, but the

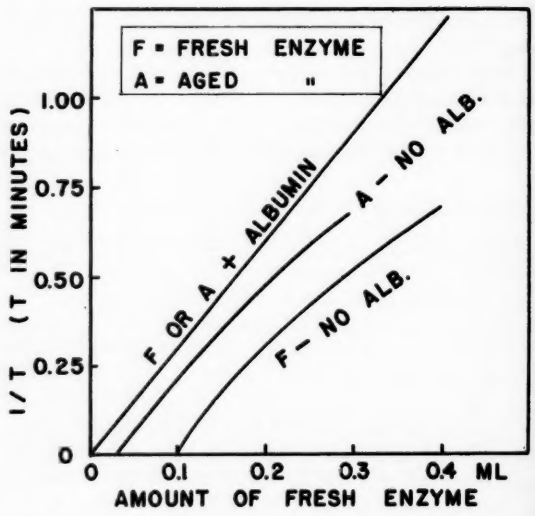

FIG. 1. The stabilizing effect of albumin on the reduction of methylene blue by freshly prepared and aged preparations of highly purified milk xanthine oxidase. The enzyme in 0.1 ml of the fresh preparation gave an oxygen uptake with hypoxanthine substrate in the presence of albumin of 14 cu mm per 10 minutes. 3.4 times as much of the aged enzyme had the same activity as the fresh enzyme in reducing methylene blue in the presence of albumin.

remainder was decolorized very slowly. 5 mg GSH, 1 mg 8HQ, and 0.01 M BP gave results similar to the albumin. 5 mg of cystine or thiourea, 300 units of catalase and 0.01 M thiosemicarbazide, OP or Fe-3 Specific Versene were slightly less active than albumin. Cysteine reduced MB rapidly in the absence of the enzyme; GSH required 5 hours, while the other substances did not reduce MB by themselves.

Although CN^- inactivates XO, it does not do so rapidly, and when added to this test system in a concentration of 0.01 to 0.001 M, it was slightly less active than the albumin in allowing rapid reduction of MB; it stabilized or protected the enzyme during the 7 to 8.5 minutes required for completion of the reaction (as compared with the 6 to 7 minutes required for the albumin test system and over one hour in the absence of albumin). The following substances were inactive: 1 mg hydroquinone, salicylic acid, benzoic acid, FAD, FMN, riboflavin, DPN, TPN, 2-methyl-1:4-naphthoquinone, or cyto c; 0.01 M Fe^{++}, ferricyanide (in tris buffer), $NaNO_3$, Na_2MoO_4, ascorbic acid or dimethylglyoxime. Semicarbazide (10^{-2} to 10^{-4} M) delayed the reduction of MB beyond that of the control tube without albumin. Using CO as the gas phase gave the same results with and without albumin as were obtained when N_2 was used.

Since SH compounds as well as cystine were effective in stabilizing the enzyme under these conditions, a possible reduction of cystine to cysteine by milk XO was investigated. The system contained enzyme, 0.75 ml hypoxanthine substrate, 0.15 ml of 0.66 M phosphate buffer pH 7.4, and 125 mg cystine in a total volume of 2

ml in a Thunberg tube under N_2. While cysteine reduced MB rapidly and nonenzymatically, the presence of cystine in the above system did not change the reduction time of added MB. Furthermore, no cysteine was formed by the above system in the presence or absence of MB when the cysteine was determined colorimetrically (194) at the end of 30 minutes incubation; MB was decolorized in one minute. Hence cystine was not capable of acting as an electron acceptor for the reduced enzyme, and the stabilization effected by cystine could probably be attributed to its amino acid structure rather than to its sulfur content.

The protection afforded by catalase in the aerobic system suggested that the inactivation of the enzyme might be attributed to a peroxide effect on the SH groups of the purified enzyme. However, this could not be true of the anaerobic effect unless the gaseous N_2 was contaminated with O_2. This was checked by utilizing N_2 purified with alkaline pyrogallol. The same delayed reduction (over 2 hours) of MB occurred with the purified N_2; albumin allowed the reduction to take place in 6.5 minutes with both the original or purified N_2. Conversely, when the N_2 gas phase contained 2% O_2, the results were essentially the same (ignoring the light blue surface layer) as when N_2 alone was used. Catalase was, therefore, another protein similar to albumin in this respect rather than a specific catalyst destroying H_2O_2.

When *p*-hydroxybenzaldehyde (0.24 mg to 24 mg) was used as the substrate with purified enzyme anaerobically, the MB was reduced in 2 and 3 minutes, respectively, in the presence and absence of albumin; the corresponding values for hypoxanthine substrate were 3 minutes and more than one hour. When both substrates were present simultaneously the decolorization times in the presence and absence of albumin were the same as those obtained with hypoxanthine alone. Thus, with aldehyde substrate no stabilizer was required (although it had some beneficial effect), but the aldehyde did not act as a stabilizer or preferred substrate when present with the hypoxanthine.

There was less need for stabilizers with xanthine as compared with hypoxanthine substrate. In the presence of albumin, 8HQ or BP the reduction time with xanthine was approximately 3 minutes, as compared with 2 minutes in the presence of GSH and 5 minutes in the absence of stabilizer; hypoxanthine substrate also gave reduction in 3 minutes in the presence of albumin and 25 minutes in its absence. In the presence of albumin, the same decolorization time (4 minutes) was obtained with *1*) hypoxanthine, *2*) hypoxanthine plus xanthine or *3*) hypoxanthine plus uric acid; in the absence of albumin the mixtures were decolorized in 5–6 minutes, while the hypoxanthine alone required over 30 minutes. Hence xanthine and uric acid helped to stabilize the enzyme.

Reduction of Cytochrome c

The reduction of cyto c by milk XO is a relatively minor reaction for this enzyme (293), but the nature of the reaction has been confusing. Horecker and Heppel (105) described a requirement for oxygen in this reaction. Morell (200) found that oxygen inhibited the reaction by forming H_2O_2 which then reoxidized the cyto c. Weber *et al.* (290) suggested that some form of bound H_2O_2 generated by the action of XO in air was the actual reductant of cyto c since free H_2O_2 could not effect this reaction. Fridovich and Handler (67) suggested that the effect of oxygen on the reduction of cyto c was due indirectly to the reduction of an inorganic iron contaminant by the uric acid formed in the reaction. Part of this confusion may be related to the purity of the XO utilized for the various studies.

The effect of various metal-binding agents on the reduction of cyto c in the presence and absence of oxygen was studied with the purified milk XO. Although albumin had only a small stimulating effect on this reaction, it was added routinely as a precautionary "stabilizer." The system was composed of milk XO, catalase, 0.01 M phosphate buffer pH 7.4, 1.8 mg of cyto c, 5 mg albumin, and 0.5 cc of 0.005 M hypoxanthine in the side arm, for a total volume of 3 cc. The tube was evacuated and filled with N_2 several times. After checking for the absence of any spontaneous reduction of cyto c by the reagents, the hypoxanthine was tipped in, and the optical density at 550 mμ was read every minute for 5 minutes. Oxygen was then bubbled through the solution intermittently and the readings were continued for an additional 5 minutes. The same measurements were repeated in the presence of 1.5 mg cystine, 0.3 cc of a saturated solution of 8HQ or BP, and in a final concentration of 0.01 M Fe-3 Specific Versene or 0.001 M KCN. Glutathione and cysteine were not studied because they reduced the cyto c directly.

The results are shown in Figure 2. In agreement with Horecker and Heppel (105) the reduction of cyto c by milk XO was 5 times faster in O_2 as compared with N_2. However, oxygen was not obligatory for this reaction since significant reduction took place in nitrogen. Moreover the anaerobic reduction was increased about 2.5 times by the addition of 8 HQ, Fe-3 Specific Versene or CN^-, and it was increased approximately 8-fold by the addition of BP. The oxygen effect was still obtained in the presence of these iron-binding agents, but they produced much less stimulation of the reaction in the presence of O_2. The rate of reduction of cyto c in air was the same in the presence or absence of 8HQ. Fe-3 Specific Versene and CN^- increased the aerobic reaction by 30 and 75 per cent, respectively, while BP increased the rate 4-fold. Cystine had no major effect on the anaerobic or aerobic reduction of cytochrome c.

The most effective stimulant of the anaerobic reduction of cyto c by milk XO, bipyridyl, reacts with Fe^{++} but has no effect on the molybdoflavoprotein, nitrate reductase. These agents, therefore, presumably exert their effect on milk XO by combining with the enzymatic Fe. The similar stimulation of this reaction by oxygen may reflect nothing more than a combination of oxygen with enzymatic Fe. Such an oxygenated ferrous-xanthine

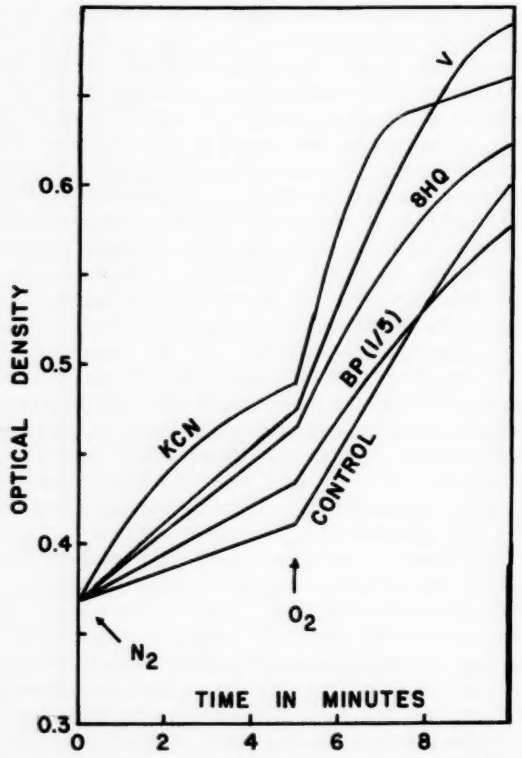

FIG. 2. The effect of iron-binding agents: α,α-bipyridyl (BP), 8-hydroxyquinoline (8HQ), Fe-3 Specific Versene (V), and KCN, on the reduction of cytochrome c by milk xanthine oxidase and hypoxanthine in N_2 and O_2. Because of the marked stimulation produced, only $\frac{1}{5}$ the amount of enzyme was used with bipyridyl as with the others.

oxidase theoretically could pass electrons on to cyto c, or the oxygen itself could be reduced to H_2O_2. The reduction of oxygen by milk XO is slower than the dehydrogenation of the substrate but is 40 times faster than the reduction of cyto c. The poor reaction of milk XO with cyto c could well be due to the fact that only the oxygenated enzyme reacts well with cyto c, but the oxygen then competes with the cyto c for electrons. An oxygenated iron structure in the enzyme is also compatible with an "oxidase group" which can be inhibited by semicarbazide and restored by iron-binding agents.

Removal of Fe From Milk Xanthine Oxidase

Attempts to remove the Fe selectively from milk XO were unsuccessful. Two treatments of the enzyme at room temperature with BP and ascorbic acid in 0.5 M acetate buffer at pH 4.5 for 24 hours, followed by repeated precipitation of the protein with 0.6 saturated ammonium sulfate, led to a progressive denaturation of the enzyme with a comparable loss of all constituents. One half to $\frac{2}{3}$ of the original activity was recovered as soluble enzyme following the first treatment, and $\frac{1}{3}$-$\frac{1}{2}$ was recovered after the second treatment. After both treatments the remaining enzyme had the same characteristics as the original enzyme. It had a normal spectrum, a normal oxidase:dehydrogenase ratio, and it contained the same relative amounts of flavin, Fe and Mo. The treatment, therefore, disrupted the entire molecule at the same time that the Fe was being removed. The inactive denatured protein precipitate which formed in this process contained little Fe or flavin, and exhibited only a relatively weak end-absorption above 300 mμ in its spectrum.

SUMMARY

1) The reaction inactivation which highly purified milk XO undergoes while oxidizing hypoxanthine substrate aerobically or anaerobically is prevented or minimized by "inert" proteins such as albumin, as well as by a variety of metal-binding agents which presumably react with the iron.

2) The oxidation of an aldehyde substrate is different because it produces relatively little inactivation anaerobically, and the very marked destruction of the enzyme which occurs during the aerobic oxidation of aldehydes is not prevented by those agents which are effective with hypoxanthine substrate.

3) The anaerobic reduction of cytochrome c by milk XO was stimulated by several iron-binding agents, especially α,α-bipyridyl. An oxygenated ferrous-xanthine oxidase was postulated as the structure responsible for the reaction with cytochrome.

4) Attempts to remove Fe selectively from milk XO by treatment with bipyridyl at pH 4.5 disrupted the molecule and caused a comparable loss of Fe, Mo, FAD and all enzymatic activities.

REFERENCES

1. ABOOD, L. G., F. A. GIBBS AND E. GIBBS. *A.M.A. Arch. Neurol. & Psychiat.* 77: 643, 1957.
2. ADELSTEIN, S. J. AND B. L. VALLEE. *J. Biol. Chem.* 233: 589, 1958.
3. ADELSTEIN, S. J. AND B. L. VALLEE. *J. Biol. Chem.* 234: 824, 1959.
4. AKERFELDT, T. *Science* 125: 117, 1957.
5. ALDERTON, G., W. H. WARD AND H. L. FEVOLD. *Arch. Biochem.* 11: 9, 1946.
6. ALLEN, T. H. AND J. H. BODINE. *Science* 94: 443, 1941.
7. ANGENENT, W. J. AND G. B. KOELLE. *Science* 116: 543, 1952.
8. APPLEBY, C. A. AND R. K. MORTON. *Nature, London* 173: 749, 1954.
9. APPLEBY, C. A. AND R. K. MORTON. *Biochem. J.* 71: 492, 1959; 73: 539, 1959.
10. ASIMOV, I. AND C. R. DAWSON. *J. Am. Chem. Soc.* 72: 820, 1950.
11. BACH, S. J., M. DIXON AND L. G. ZERFAS. *Biochem. J.* 40: 229, 1946.
12. BAUM, H., G. HUBSCHER AND H. R. MAHLER. *Biochim. et biophys. acta* 22: 514, 1956.
13. BAXTER, C. F. AND R. VAN REEN. *Biochim. et biophys. acta* 28: 575, 1958.
14. BAYER, E. AND K. H. HAUSER. *Experientia* 11: 254, 1955.
15. BEARN, A. G. AND H. G. KUNKEL. *J. Lab. & Clin. Med.* 45: 623, 1955.

16. Behm, R. C. and J. M. Nelson. *J. Am. Chem. Soc.* 66: 711, 1944.
17. Berfenstam, R. *Acta paediat.* 41: Suppl. 87, 1952.
18. Bergel, F. and R. C. Bray. *Biochem. J.* 73: 182, 1959.
19. Bernheim, F. and M. L. C. Bernheim. *J. Biol. Chem.* 145: 213, 1942.
20. Bickel, H., H. E. Schultze, W. Gruter and I. Gollner. *Klin. Wchnschr.* 34: 961, 1956.
21. Bielig, H. J. and E. Bayer. *Naturwiss.* 42: 125, 466, 1955.
22. Bloch, B. *Am. J. M. Sc.* 177: 609, 1929.
23. Boeri, E., E. Cutolo, M. Luzzati and L. Tosi. *Arch. Biochem. Biophys.* 56: 487, 1955.
24. Boeri, E. and L. Tosi. *Arch. Biochem. Biophys.* 60: 463, 1956.
25. Bray, R. C. *Biochem. J.* 73: 690, 1959.
26. Bray, R. C., B. G. Malmström and T. Vånngard. *Biochem. J.* 73: 193, 1959.
27. Broman, L. *Nature, London* 182: 1655, 1958.
28. Brown, F. C. and D. N. Ward. *J. Am. Chem. Soc.* 79: 2647, 1957.
29. Bush, J. A., J. P. Mahoney, C. J. Gubler, G. E. Cartwright and M. M. Wintrobe. *J. Lab. & Clin. Med.* 47: 898, 1956.
30. Cartwright, G. E., R. E. Hodges, C. J. Gubler, J. P. Mahoney, K. Daum, M. M. Wintrobe and W. B. Bean. *J. Clin. Invest.* 33: 1487, 1954.
31. Cohn, E. J. *Blood* 3: 471, 1948.
32. Cotzias, G. C. *Physiol. Rev.* 38: 503, 1958.
33. Cotzias, G. C., D. C. Borg and A. J. Bertinchamps. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 50.
34. Crandall, D. I. *J. Biol. Chem.* 212: 565, 1955.
35. Crandall, D. I., K. Yasunobu, R. C. Krueger and H. S. Mason. *Fed. Proc.* 17: 207, 1958.
36. Cunningham, L. W., R. L. Fischer and C. S. Vestling. *J. Am. Chem. Soc.* 77: 5703, 1955.
37. Curdel, A., L. Naslin and F. Labeyrie. *Compt. rend. acad. sci.* 249: 1959, 1959.
38. Curzon, G. *Nature, London* 181: 115, 1958.
39. Curzon, G. and L. Vallet. *Biochem. J.* 74: 279, 1960.
40. Dalton, H. R. and J. M. Nelson. *J. Am. Chem. Soc.* 61: 2946, 1939.
41. Davidson, J. N. *Biochem. J.* 36: 252, 1942.
42. Davis, R. P. *J. Am. Chem. Soc.* 81: 5674, 1959.
43. Dawson, C. R. and M. F. Mallette. *Advances Protein Chem.* 2: 179, 1945.
44. Dawson, C. R. and W. B. Tarpley. In: *The Enzymes*, (vol. II, pt. 1), edited by J. B. Sumner and K. Myrbäck. New York: Acad. Press, 1951, p. 454.
45. Demulder, R. *A.M.A. Arch. Int. Med.* 102: 254, 1958.
46. De Renzo, E. C. *Advances Enzymol.* 17: 293, 1956.
47. Dewan, J. G. *Biochem. J.* 32: 1378, 1938.
48. Doisy, R. J., D. A. Richert and W. W. Westerfeld. *J. Biol. Chem.* 217: 307, 1955.
49. Drabkin, D. L. *Physiol. Rev.* 31: 345, 1951.
50. Dreyfus, J. C. and G. Schapira. *Bull soc. chim. biol.* 37: 541, 1955.
51. Du Bois, K. P. and W. F. Erway. *J. Biol. Chem.* 165: 711, 1946.
52. Du Buy, H. G., M. W. Woods, D. Burk and M. D. Lackey. *J. Nat. Cancer Inst.* 9: 325, 1948.
53. Dunn, F. J. and C. R. Dawson. *J. Biol. Chem.* 189: 485, 1951.
54. Ehrenberg, A. and C. B. Laurell. *Acta chem. scandinav.* 9: 68, 1955.
55. Eichenberger, E. and K. V. Thimann. *Arch. Biochem. Biophys.* 67: 466, 1957.
56. Farrant, J. L. *Biochim. et biophys. acta* 13: 569, 1954.
57. Felsenfeld, G. *J. Cell & Comp. Physiol.* 43: 23, 1954.
58. Fiala, S. and D. Burk. *Arch. Biochem.* 20: 172, 1949.
59. Fitzpatrick, T. B. *A.M.A. Arch. Dermat. & Syph.* 65: 379, 1952.
60. Fitzpatrick, T. B., S. W. Becker, Jr., A. B. Lerner and H. Montgomery. *Science* 112: 223, 1950.
61. Fitzpatrick, T. B., A. B. Lerner, E. Calkins and W. H. Summerson. *Proc. Soc. Exper. Biol. & Med.* 75: 394, 1950.
62. Foster, M. and R. Cook, Jr. *Proc. Soc. Exper. Biol. & Med.* 85: 120, 1954.
63. Fraenkel-Conrat, H. *Arch. Biochem.* 28: 452, 1950.
64. Fraenkel-Conrat, H. and R. E. Feeney. *Arch. Biochem.* 29: 101, 1950.
65. Fraenkel-Conrat, H. and R. R. Porter. *Biochim. et biophys. acta* 9: 557, 1952.
66. Fridovich, I. and P. Handler. *J. Biol. Chem.* 231: 899, 1958.
67. Fridovich, I. and P. Handler. *J. Biol. Chem.* 233: 1581, 1958.
68. Frieden, C. *Biochim. et biophys. acta* 27: 431, 1958.
69. Frieden, C. *J. Biol. Chem.* 234: 809, 815, 1959.
70. Frieden, E. *Biochim. et biophys. acta* 9: 696, 1952.
71. Frieden, E. and I. W. Maggiolo. *Biochim. et biophys. acta* 24: 42, 1957.
72. Frieden, E. and B. Naile. *Arch. Biochem. Biophys.* 48: 448, 1954.
73. Geller, E., S. Eiduson and A. Yuwiler. *J. Neurochem.* 5: 73, 1959.
74. Gitlin D. and C. A. Janeway. *Nature, London* 185: 693, 1960.
75. Glenn, J. L. and F. L. Crane. *Biochim. et biophys. acta* 22: 111, 1956.
76. Granick, S. *Chem. Rev.* 38: 379, 1946.
77. Granick, S. *Physiol. Rev.* 31: 489, 1951.
78. Granick, S. and P. F. Hahn. *J. Biol. Chem.* 155: 661, 1944.
79. Granick, S. and L. Michaelis. *Science* 95: 439, 1942.
80. Graubard, M. *Enzymologia* 5: 332, 1939.
81. Green, S., A. Mazur and E. Shorr. *J. Biol. Chem.* 220: 237, 1956.
82. Greenstein, J. P., J. Werne, A. B. Eschenbrenner and F. M. Leuthardt. *J. Nat. Cancer Inst.* 5: 55, 1944.
83. Gregg, D. C. and W. H. Miller. *J. Am. Chem. Soc.* 62: 374, 1940.
84. Gregg, D. C. and J. M. Nelson. *J. Am. Chem. Soc.* 62: 2500, 1940.
85. Gryder, R. M., J. S. Friedenwald and C. Carlson. *Arch. Biochem. Biophys.* 54: 281, 1955.
86. Gubler, C. J., M. E. Lahey, G. E. Cartwright and M. M. Wintrobe. *J. Clin. Invest.* 32: 405, 1953.
87. Gurd, F. R. N. and D. S. Goodman. *J. Am. Chem. Soc.* 74: 670, 1952.
88. Gurd, F. R. N. and P. E. Wilcox. *Advances Protein Chem.* 11: 311, 1956.
89. Gutfreund, H. and J. M. Sturtevant. *Biochem. J.* 73: 1, 1959.
90. Hampton, J. K., Jr. and J. B. Kahn, Jr. *Am. J. Physiol.* 174: 226, 1953.
91. Hegsted, D. M., J. M. McKibbin and C. K. Drinker. *U.S.P.H. Reports*, Suppl. 179, 1945.
92. Hesselbach, M. L. *J. Nat. Cancer Inst.* 12: 337, 1951.
93. Heymann, H., Z. Rogach and R. L. Mayer. *J. Am. Chem. Soc.* 76: 6330, 1954.
94. Higgins, E. S., D. A. Richert and W. W. Westerfeld. *J. Nutr.* 59: 539, 1956.
95. Hoch, F. L. and B. L. Vallee. In: *Trace Elements*, edited by C. A. Lamb, O. G. Bentley and J. M. Beattie. New York: Acad. Press, 1958, p. 337.
96. Hoch, F. L., R. J. P. Williams and B. L. Vallee. *J. Biol. Chem.* 232: 453, 1958.
97. Hofstee, B. H. J. *J. Biol. Chem.* 216: 235, 1955.
98. Hofstee, B. H. J. *Arch. Biochem. Biophys.* 59: 352, 1955.
99. Hogeboom, G. H. and M. H. Adams. *J. Biol. Chem.* 145: 273, 1942.
100. Holmberg, C. G. *Biochem. J.* 33: 1901, 1939.
101. Holmberg, C. G. and C. B. Laurell. *Acta chem. scandinav.* 1: 944, 1947.
102. Holmberg, C. G. and C. B. Laurell. *Acta chem. candinav.* 2: 550, 1948.

103. Holmberg, C. G. and C. B. Laurell. *Acta chem. scandinav.* 5: 476, 1951.
104. Horecker, B. L. *J. Biol. Chem.* 183: 593, 1950.
105. Horecker, B. L. and L. A. Heppel. *J. Biol. Chem.* 178: 683, 1949.
106. Hove, E., C. A. Elvehjem and E. B. Hart. *J. Biol. Chem.* 136: 425, 1940.
107. Hubscher, G., H. Baum and H. R. Mahler. *Biochim. et biophys. acta* 23: 43, 1957.
108. Huff, R. L., T. G. Hennessy, R. E. Austin, J. F. Garcia, B. M. Roberts and J. H. Lawrence. *J. Clin. Invest.* 29: 1041, 1950.
109. Ingraham, L. L. *J. Am. Chem. Soc.* 77: 2875, 1955.
110. Ingraham, L. L. *J. Am. Chem. Soc.* 78: 5095, 1956.
111. Isaka, S. *Nature, London* 179: 578, 1957.
112. Jager, B. V. *J. Clin. Invest.* 28: 792, 1949.
113. James, W. O., E. A. H. Roberts, H. Beevers and P. C. DeKock. *Biochem. J.* 43: 626, 1948.
114. Joselow, M. and C. R. Dawson. *J. Biol. Chem.* 191: 1, 11, 1951.
115. Joselow, M. and C. R. Dawson. *Science* 121: 300, 1955.
116. Joubert, F. J. *Biochim. et biophys. acta* 14: 127, 1954.
117. Kaplan, N. O., M. M. Ciotti and F. E. Stolzenbach. *J. Biol. Chem.* 211: 419, 1954.
118. Kearney, E. B. and T. P. Singer. *Biochim. et biophys. acta* 17: 596, 1955.
119. Keilin, D. and T. Mann. *Proc. Roy. Soc., London, ser. B.* 125: 187, 1938.
120. Keilin, D. and T. Mann. *Nature, London* 143: 23, 1939.
121. Keilin, D. and T. Mann. *Nature, London* 145: 304, 1940.
122. Keilin, D. and T. Mann. *Biochem. J.* 34: 1163, 1940.
123. Keilin, D. and E. F. Hartree. *Proc. Roy. Soc., London, ser. B.* 119: 114, 1936.
124. Keller, H. and P. Gottwald. *Hoppe-Seyler's Ztschr. physiol. Chem.* 318: 201, 1960.
125. Keller, H. and U H. Peters. *Hoppe-Seyler's Ztschr. physiol. Chem.* 317: 228, 1959.
126. Kendal, L. P. *Biochem. J.* 44: 442, 1949.
127. Kertesz, D. *Bull. soc. chim. biol.* 32: 587, 1950.
128. Kertesz, D. *Bull. soc. chim. biol.* 33: 1400, 1951.
129. Kertesz, D. *Biochim. et biophys. acta* 9: 170, 1952.
130. Kertesz, D. *J. Nat. Cancer Inst.* 14: 1081, 1093, 1954.
131. Kertesz, D. *Nature, London* 180: 506, 1957.
132. Kertesz, D. and R. Zito. *Nature, London* 179: 1017, 1957.
133. Kimmel, J. R., H. Markowitz and D. M. Brown. *J. Biol. Chem.* 234: 46, 1959.
134. Kinsky, S. C. and W. D. McElroy. *Arch. Biochem. Biophys.* 73: 466, 1958.
135. Klotz, I. M. and R. E. Heiney. *Proc. Nat. Acad. Sc. U.S.* 43: 717, 1957.
136. Klotz, I. M. and T. A. Klotz. *Science* 121: 477, 1955.
137. Knox, W. E. and S. W. Edwards. *J. Biol. Chem.* 216: 479, 1955.
138. Koechlin, B. A. *J. Am. Chem. Soc.* 74: 2649, 1952.
139. Kohn, R. R. *Enzymologia* 17: 193, 1955.
140. Krueger, R. C. *J. Am. Chem. Soc.* 72: 5582, 1950.
141. Kubo, H., M. Iwatsubo, H. Watari and T. Soyama. *J. Biochem. (Jap.)* 46: 1171, 1959.
142. Kubowitz, F. *Biochem. Ztschr.* 299: 32, 1938; 296: 443, 1938; 292: 221, 1937.
143. Kukita, A. and T. B. Fitzpatrick. *Science* 121: 893, 1955.
144. Kull, F. C., M. R. Grimm and R. L. Mayer. *Proc. Soc. Exper. Biol. & Med.* 86: 330, 1954.
145. Lamb, C. A., O. G. Bentley and J. M. Beattie, Eds. *Trace Elements.* New York: Acad. Press, 1958.
146. Lamboov, J. P. *J. Am. Chem. Soc.* 78: 771, 1956.
147. Lauffer, M. A. and L. G. Swaby. *Biol. Bull.* 108: 290, 1955.
148. Laurell, C. B. *Acta physiol. scandinav.* 14: Suppl. 46, 1947.
149. Laurell, C. B. *Blood* 6: 183, 1951.
150. Laurell, C. B. *Phamacol. Rev.* 4: 371, 1952.
151. Laurell, C. B. *Acta chem. scandinav.* 7: 1407, 1953.
152. Laurell, C. B. and B. Ingelman. *Acta chem. scandinav.* 1: 770, 1947.
153. Leach, B. E., M. Cohen, R. G. Heath and S. Martens. *A.M.A. Arch. Neurol. & Psychiat.* 76: 635, 1956.
154. Lemberg, R. and J. W. Legge. *Hematin Compounds and Bile Pigments.* New York: Interscience, 1949.
155. Lerner, A. B. *Advances Enzymol.* 14: 73, 1953.
156. Lerner, A. B. and T. P. Fitzpatrick. *Physiol. Rev.* 30: 91, 1950.
157. Lerner, A. B., T. B. Fitzpatrick, E. Calkins and W. H. Summerson. *J. Biol. Chem.* 178: 185, 1949; 187: 793, 1950; 191: 799, 1951.
158. Lillie, R. D. *J. Histochem.* 4: 318, 1956.
159. Lindskog, S. *Biochim. et biophys. acta* 39: 218, 1960.
160. Lindskog, S. and B. G. Malmström. *Biochem. & Biophys. Res. Comm.* 2: 213, 1960.
161. Loewus, M. W. and R. A. Fineberg. *Biochim. et biophys. acta* 26: 441, 1957.
162. Loftfield, R. B. and R. Bonnichsen. *Acta chem. scandinav.* 10: 1547, 1956.
163. Lontie, R. *Clin. Chim. Acta* 3: 68, 1958. (Chem. Abstracts 52: 8283, 1958).
164. Lorincz, A. L. *J. Invest. Dermat.* 15: 425, 1950.
165. Lovett-Janison, P. L. and J. M. Nelson. *J. Am. Chem. Soc.* 62: 1409, 1940.
166. Mahler, H. R. *Advances Enzymol.* 17: 233, 1956.
167. Mahler, H. R. In: *Trace Elements,* edited by C. A. Lamb, O. G. Bentley and J. M. Beattie. New York: Acad. Press, 1958, p. 311.
168. Mahler, H. R. and D. G. Elowe. *J. Biol. Chem.* 210: 165, 1954.
169. Mahler, H. R. and D. E. Green. *Science* 120: 7, 1954.
170. Mahler, H. R., G. Hubscher and H. Baum. *J. Biol. Chem.* 216: 625, 1955.
171. Mahler, H. R., B. Mackler, D. E. Green and R. M. Bock. *J. Biol. Chem.* 210: 465, 1954.
172. Mahler, H. R., N. K. Sarkar, L. P. Vernon and R. A. Alberty. *J. Biol. Chem.* 199: 585, 1952.
173. Mallette, M. F. and C. R. Dawson. *Arch. Biochem.* 23: 29, 1949.
174. Malmström, B. G., G. Fahraeus and R. Mosbach. *Biochim. et biophys. acta* 28: 652, 1958.
175. Malmström, B. G., R. Mosbach and T. Vånngard. *Nature, London* 183: 321, 1959.
176. Mann, T. and D. Keilin. *Proc. Roy. Soc., London, ser. B.* 126: 303, 1938.
177. Mann, T. and D. Keilin. *Nature, London* 146: 164, 1940.
178. Markowitz, H., G. E. Cartwright and M. M. Wintrobe. *J. Biol. Chem.* 234: 40, 1959.
179. Mason, H. S. *Advanced Enzymol.* 16: 105, 1955.
180. Mason, H. S. *Nature, London* 177: 79, 1956.
181. Mason, H. S. *Advances Enzymol.* 19: 79, 1957.
182. Mason, H. S., W. L. Fowlks and E. Peterson. *J. Am. Chem. Soc.* 77: 2914, 1955.
183. Massey, V. *J. Biol. Chem.* 229: 763, 1957.
184. Massey, V. *Biochim. et biophys. acta* 30: 500, 1958.
185. Mathies, J. C. *J. Biol. Chem.* 233: 1121, 1958.
186. Mayer, A. M. *Nature, London* 180: 1417, 1957.
187. Mayer, R. L., M. R. Grimm and F. C. Kull. *Proc. Soc. Exper. Biol. & Med.* 81: 163, 1952.
188. Mazur, A., S. Baez and E. Shorr. *J. Biol. Chem.* 213: 147, 1955.
189. Mazur, A., S. Green, A. Saha and A. Carleton. *J. Clin. Invest.* 37: 1809, 1958.
190. Mazur, A., S. Green and E. Shorr. *J. Biol. Chem.* 220: 227, 1956.
191. Mazur, A., I. Litt and E. Shorr. *J. Biol. Chem.* 187: 485, 1950.
192. McCarthy, J. F., L. F. Green and C. G. King. *J. Biol. Chem.* 128: 455, 1939.
193. McElroy, W. D. and B. Glass (editors). *Copper Metabolism.* Baltimore: Johns Hopkins Press, 1950.

194. MECHAM, D. K. *J. Biol. Chem.* 151: 643, 1943.
195. MEIKLEJOHN, G. T. AND C. P. STEWART. *Biochem. J.* 35: 755, 1941.
196. MICHAELIS, L. *Advances Prot. Chem.* 3: 53, 1947.
197. MICHAELIS, L., C. D. CORYELL AND S. GRANICK. *J. Biol. Chem.* 148: 463, 1943.
198. MILLIKAN, G. A. *Physiol. Rev.* 19: 503, 1939.
199. MOHAMED, M. S. AND D. M. GREENBERG. *J. Gen. Physiol.* 37: 433, 1954.
200. MORELL, D. B. *Biochem. J.* 51: 666, 1952.
201. NAKAMURA, T. *Biochim. et biophys. acta* 30: 44, 1958.
202. NASON, A. AND H. J. EVANS. *J. Biol. Chem.* 202: 655, 1953.
203. NELSON, J. M. AND C. R. DAWSON. *Advances Enzymol.* 4: 99, 1944.
204. NICHOLAS, D. J. D. AND A. NASON. *J. Biol. Chem.* 207: 353, 1954.
205. NICHOLAS, D. J. D. AND A. NASON. *J. Biol. Chem.* 211: 183, 1954.
206. NICHOLAS, D. J. D. AND H. M. STEVENS. *Nature, London* 176: 1066, 1955.
207. OLSON, J. A. AND C. B. ANFINSEN. *J. Biol. Chem.* 202: 841, 1953.
208. PASCHKIS, K. E., A. CANTAROW, W. M. HART AND A. E. RAKOFF. *Proc. Soc. Exper. Biol. & Med.* 57: 37, 1944.
209. PATTERSON, E. L., R. MILSTREY AND E. L. R. STOKSTAD. *Proc. Soc. Exper. Biol. & Med.* 95: 617, 1957.
210. PERRY, H. M., JR. AND E. F. PERRY. *J. Clin. Invest.* 38: 1452, 1959.
211. PERRY, H. M., JR. AND G. H. CAMEL. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 209.
212. PFLEIDERER, G., D. JECKEL AND T. WIELAND. *Biochem. Ztschr.* 330: 296, 1958.
213. PINSENT, J. *Biochem. J.* 57: 10, 1954.
214. PORTER, H. AND S. AINSWORTH. *Proc. Soc. Exper. Biol. & Med.* 98: 277, 1958.
215. PORTER, H. AND S. AINSWORTH. *J. Neurochem.* 5: 91, 1959.
216. PORTER, H. AND J. FOLCH. *J. Neurochem.* 1: 260, 1957.
217. PORTER, H. AND J. FOLCH. *A.M.A. Arch. Neurol. Psychiat.* 77: 8, 1957.
218. PORTER, C. C., D. C. TITUS, B. E. SANDERS AND E. V. C. SMITH. *Science* 126: 1014, 1957.
219. POWERS, W. H. AND C. R. DAWSON. *J. Gen. Physiol.* 27: 181, 1944.
220. POWERS, W. H., S. LEWIS AND C. R. DAWSON. *J. Gen. Physiol.* 27: 167, 1944.
221. RATH, C. E. AND C. A. FINCH. *J. Clin. Invest.* 28: 79, 1949.
222. REDMOND, J. R. *J. Cell. & Comp. Physiol.* 46: 209, 1955.
223. RICHERT, D. A. AND W. W. WESTERFELD. *J. Biol. Chem.* 209: 179, 1954.
224. RICHTER, G. W. *J. Biophys. Biochem. Cytol.* 4: 55, 1958.
225. SCHADE, A. L. AND L. CAROLINE. *Science* 100: 14, 1944; 104: 340, 1946.
226. SCHADE, A. L., R. W. REINHART AND H. LEVY. *Arch. Biochem.* 20: 170, 1949.
227. SCHEINBERG, I. H. AND A. G. MORELL. *J. Clin. Invest.* 36: 1193, 1957.
228. SCHEINBERG, I. H., A. G. MORELL, R. S. HARRIS AND A. BERGER. *Science* 126: 925, 1957.
229. SCHEPARTZ, B. *J. Biol. Chem.* 205: 185, 1953.
230. SCHORR, E. *Harvey Lectures* 50: 112, 1954–55.
231. SCHROEDER, H. A. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 59.
232. SCHWARZ, K., J. G. BIERI, G. M. BRIGGS AND M. L. SCOTT. *Proc. Soc. Exper. Biol. & Med.* 95: 621, 1957.
233. SCHWARZ, K. AND C. M. FOLTZ. *J. Am. Chem. Soc.* 79: 3292, 1957.
234. SCHWARZ, K. AND W. MERTZ. *Arch. Biochem. Biophys.* 85: 292, 1959.
235. SEVEN, M. J. (editor). *Metal-Binding in Medicine*. Philadelphia: Lippincott, 1960, p. 81.
236. SEVEN, M. J. (editor). *Metal-Binding in Medicine*. Philadelphia: Lippincott, 1960, pp. 95, 146, 230.
237. SHIELDS, G. S., H. MARKOWITZ, G. E. CARTWRIGHT AND M. M. WINTROBE. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 259.
238. SHODEN, A., B. W. GABRIO AND C. A. FINCH. *J. Biol. Chem.* 204: 823, 1953.
239. SILVERBLATT, E. AND C. G. KING. *Enzymologia* 2: 222, 1938.
240. SINGER, T. P. AND E. B. KEARNEY. In: *The Proteins*, vol. 2, pt. A, edited by H. Neurath and K. Bailey. New York: Acad. Press, 1954, p. 123.
241. SINGER, T. P., E. B. KEARNEY AND P. BERNATH. *J. Biol. Chem.* 223: 599, 1956.
242. SINGER, T. P., E. B. KEARNEY AND V. MASSEY. *Arch. Biochem. Biophys.* 60: 255, 1956.
243. SINGER, T. P., E. B. KEARNEY AND V. MASSEY. *Advances Enzymol.* 18: 65, 1957.
244. SINGER, T. P., E. B. KEARNEY AND N. ZASTROW. *Biochem. Biophys. Acta* 17: 154, 1955.
245. SINGER, T. P., V. MASSEY AND E. B. KEARNEY. *Arch. Biochem. Biophys.* 69: 405, 1957.
246. SMITH, E. AND H. T. HANSON. *J. Biol. Chem.* 179: 803, 1949.
247. SPENCER, R. P. AND J. B. FIELD. *Proc. Soc. Exper. Biol. & Med.* 88: 576, 1955.
248. SREERANGACHAR, H. B. *Biochem. J.* 37: 667, 1943.
249. STOTZ, E. *J. Biol. Chem.* 133: C 1940.
250. STOTZ, E., C. J. HARRER AND C. G. KING. *J. Biol. Chem.* 119: 511, 1937.
251. SUDA, M. AND Y. TAKEDA. *J. Biochem. (Jap)* 37: 381, 1950.
252. SUMNER, J. B. AND K. MYRBÄCK. *The Enzymes*, vol. II, pt. I. New York: Acad. Press, 1951, pp. 357, 397, 428.
253. SURGENOR, D. M., B. A. KOECHLIN AND L. E. STRONG. *J. Clin. Invest.* 28: 73, 1949.
254. TANAKA, S. *J. Biochem. (Jap)* 37: 129, 1950.
255. TANFORD, C. AND J. EPSTEIN. *J. Am. Chem. Soc.* 76: 2170, 1954.
256. TAUBER, H. AND I. S. KLEINER. *Proc. Soc. Exper. Biol. & Med.* 32: 577, 1935.
257. TENENBAUM, L. E. AND H. JENSEN. *J. Biol. Chem.* 147: 27, 1943.
258. THEORELL, H. *Advances Enzymol.* 7: 265, 1947.
259. THEORELL, H., M. BEZNAK, R. BONNICHSEN, K. PAUL AND Å. ÅKESON. *Acta chem. scand.* 5: 445, 1951.
260. TIMASHEFF, S. N. AND I. TINOCO, JR. *Arch. Biochem. Biophys.* 66: 427, 1957.
261. TISSIERES, A. *Nature, London* 162: 340, 1948.
262. TOKUYAMA, K. *J. Biochem. (Jap)* 46: 1379, 1453, 1959.
263. TOMPSETT, S. L. *Biochem. J.* 34: 959, 961, 1940.
264. TRUBOWITZ, S., D. FELDMAN, C. BENANTE AND D. KIRMAN. *Proc. Soc. Exper. Biol. & Med.* 95: 35, 1957.
265. TUPPER, R., R. W. E. WATTS AND A. WORMALL. *Biochem. J.* 50: 429, 1951.
266. UNDERWOOD, E. J. *Trace Elements in Human and Animal Nutrition*. New York: Acad. Press, 1956.
267. VALENTINE, W. N., K. TANAKA AND R. E. FREDRICKS. *J. Lab. & Clin. Med.* 55: 303, 1960.
268. VALLEE, B. L. *Advances Protein Chem.* 10: 317, 1955.
269. VALLEE, B. L. *Physiol. Rev.* 39: 443, 1959.
270. VALLEE, B. L. AND T. L. COOMBS. *J. Biol. Chem.* 234: 2615, 1959.
271. VALLEE, B. L., T. L. COOMBS AND R. J. P. WILLIAMS. *J. Am. Chem. Soc.* 80: 397, 1958.
272. VALLEE, B. L. AND F. L. HOCH. *Proc. Nat. Acad. Sc. U. S.* 41: 327, 1955.
273. VALLEE, B. L. AND F. L. HOCH. *J. Biol. Chem.* 225: 185, 1957.
274. VALLEE, B. L., F. L. HOCH, S. J. ADELSTEIN AND W. E. C. WACKER. *J. Am. Chem. Soc.* 78: 5879, 1956.
275. VALLEE, B. L., F. L. HOCH AND W. L. HUGHES, JR. *Arch. Biochem. Biophys.* 48: 347, 1954.
276. VALLEE, B. L., J. H. R. KÄGI AND F. L. HOCH. *Fed. Proc.* 17: 326, 1958.

277. VALLEE, B. L., H. D. LEWIS, M. D. ALTSCHULE AND J. G. GIBSON II. *Blood* 4: 467, 1949.
278. VALLEE, B. L. AND H. NEURATH. *J. Biol. Chem.* 217: 253, 1955.
279. VALLEE, B. L., J. A. RUPLEY, T. L. COOMBS AND H. NEURATH. *J. Biol. Chem.* 235: 64, 1960.
280. VALLEE, B. L. AND W. E. C. WACKER. *J. Am. Chem. Soc.* 78: 1771, 1956.
281. VALLEE, B. L., R. J. P. WILLIAMS AND F. L. HOCH. *J. Biol. Chem.* 234: 2621, 1959.
282. VERNON, L. P., H. R. MAHLER AND N. K. SARKAR. *J. Biol. Chem.* 199: 599, 1952.
283. VIKBLADH, I. *Scandinav. J. Clin. & Lab. Invest.*, Suppl. 2, 3: 1951.
284. VOLKMANN, C. M. AND E. BEERSTECHER, JR. *J. Biol. Chem.* 223: 1021, 1956.
285. WALLENFELS, K., H. SUND, A. FAESSLER AND W. BURCHARD. *Biochem. Ztschr.* 329: 31, 1957.
286. WALSHE, J. M. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 265.
287. WARNER, R. C. AND I. WEBER. *J. Biol. Chem.* 191: 173, 1951.
288. WARNER, R. C. AND I. WEBER. *J. Am. Chem. Soc.* 75: 5094, 1953.
289. WEBB, E. C. AND R. VAN HEYNINGEN. *Biochem. J.* 41: 74, 1946.
290. WEBER, M. M., H. M. LENHOF AND N. O. KAPLAN. *J. Biol. Chem.* 220: 93, 1956.
291. WESTERFELD, W. W., D. A. RICHERT AND R. J. BLOOM. *J. Biol. Chem.* 234: 1889, 1959.
292. WESTERFELD, W. W., D. A. RICHERT AND E. S. HIGGINS. In: *Inorganic Nitrogen Metabolism*, edited by W. D. McElroy and B. Glass. Baltimore: Johns Hopkins Press, 1956, p. 492.
293. WESTERFELD, W. W., D. A. RICHERT AND E. S. HIGGINS. *J. Biol. Chem.* 234: 1897, 1959.
294. WILLIAMS, R. J. P., F. L. HOCH AND B. L. VALLEE. *J. Biol. Chem.* 232: 465, 1958.
295. WINTROBE, M. M., G. E. CARTWRIGHT AND C. J. GUBLER. *J. Nutr.* 50: 395, 1953.
296. WOLFF, H. P. *Klin. Wchnschr.* 34: 409, 1956.
297. ZUCKERKANDL, E. *Compt. rend. soc. biol.* 145: 291, 293, 1951.
298. ZUCKERKANDL, E. *Compt. rend. acad. sci.* 249: 568, 1959.

GENERAL DISCUSSION

DR. SCHWARZ: I just wonder whether you happen to know, Dr. Oser, how much of the EDTA in these rations is really absorbed by the rat. Sometime ago, after we found the effect of chromium on glucose tolerance, I asked you whether you had any information on the behavior of these animals with respect to glucose tolerance. Has it been possible to carry out glucose tolerance tests since that time?

DR. OSER: As far as your first question is concerned, we have no observations on the actual absorption of EDTA in these experiments. The glucose tolerance suggestion came about two years after these animals had been sacrificed, so it was not possible to run these tests. The blood sugar levels, which were done frequently, indicated no change. Of course this does not mean there may not have been a change in the rate at which glucose was absorbed and utilized. As far as the variation between 6.8 and 8.4 rats per litter is concerned, this is normal; seven animals per litter is not unusual. In one of these groups the two generations at the highest level had 10.5 animals per litter which is a little high as an average for something like 40 litters, but these variations are not abnormal.

DR. PHILIPS: In the course of a two-year experiment you must have seen some tumors appearing in your rats. In view of the current interest in the possibility that metal complexes may be carcinogenic it would be valuable to hear something of your findings. Perhaps you would care to comment on the belief that iron complexes are carcinogenic.

DR. OSER: Of course your comment would have much more significance than mine on that point. In these particular groups there was one subcutaneous tumor observed grossly in the male controls and none in the 250 mg/kg group at two years. There were three tumors, one subcutaneous and two mammary, observed in the controls and one subcutaneous tumor in the females at 250 mg/kg. Histopathologically, these were identified as a neurofibroma, 2 fibroadenomas, and 3 reticulum cell sarcomas. There were just one or two of each type in these groups of 25. I cannot comment specifically on the carcinogenesis of iron EDTA. In chronic carcinogenesis studies one usually has to use very large numbers of animals to determine whether a response is causally related to dosage. From our own observations I would strongly doubt that the oral administration of EDTA would induce such an effect.

DR. PHILIPS: Some notoriety has been attached to claims that iron dextran complexes are carcinogenic (Haddow, A. and E. S. Horning. *J. Nat. Cancer Inst.* 24: 109, 1960; Richmond, H. G. *Brit. Med. J.* 1: 947, 1959). One in particular, the drug "Imferon" has been withdrawn from the American market as a result. The alleged carcinogenicity of iron dextran complexes is based on the finding of local sarcomas after repeated injections of large amounts of the substance into the subcutaneous tissues of laboratory rodents. Certain cautions must be considered in interpreting such findings. These have been summarized in a recent publication (*Problems in the Evaluation of Carcinogenic Hazard from Use of Food Additives* 1960. Publication #749, National Academy of Sciences-National Research Council, Washington, D. C., p. 27) from which the following quotation is taken: "Both rats and mice frequently develop sarcomas following the subcutaneous injection of a wide variety of substances, with the subcutaneous tissue of the rat being the more sensitive. As a result, the repeated subcutaneous injection of test substances in rats and mice is often employed to detect carcinogenic activity. Parenteral injection has been recommended by Boyland as "more stringent and desirable" than oral administration for testing food additives. However, in so far as this recommendation applies to repeated subcutaneous injection, it does not withstand a critical appraisal. In the first place, some substances normally found in the body and in (and even essential to) a normal diet, e.g., glucose, fructose, and sodium chloride, have produced sarcomas in the subcutaneous tissues of rats and mice when large doses were injected repeatedly. Secondly, there are several characteristics of the subcutaneous test for carcinogenicity which throw doubt on its usefulness. The production of sar-

comas in the rat by repeated subcutaneous injection of hypertonic solutions of glucose and sodium chloride raises the strong suspicion that purely physical factors may be responsible for induction of tumors by this route. Certain substances known to be carcinogenic when ingested (azo dyes, β-napthylamine, acetylaminofluorene) produce low incidences of sarcomas when injected and are usually less effective at other sites than when given orally. Furthermore, trauma and regeneration are produced by each injection and may be of significance if repeated injections are necessary to produce the sarcomas.

Hence, it is our opinion that, when large doses of material must be given repeatedly, the subcutaneous route of testing substances for their carcinogenic activity is of limited value and of dubious interpretation."

DR. OSER: I didn't think we were likely to get into this subject, but since we have, I think for this particular audience it may be worth recalling the association of arsenic with skin cancer. Historically, if you look through the literature going back over 100 years, there appears to be an association of arsenicism with palmar-plantar hyperkeratosis which ultimately may develop into benign and then malignant skin tumors. Now, this evidence has been questioned seriously recently, at least in so far as it relates to organic arsenicals. It has not been possible, at least so far as I know, to induce skin cancers in animals by the administration of arsenic. Moreover, there has been such a vast human experience with organic arsenicals in the last 40 or 50 years, with no apparent association with cancer of the skin or elsewhere that the earlier evidence should be scrutinized more carefully. One author has observed that one of the first reported effects of arsenic poisoning in cattle was a peculiar kind of weakness and buckling of the fore legs, specific apparently in cows but not in horses. This effect has been shown recently to be attributed to selenium toxicity which has a similar specificity and similar symptoms. Whether these early observations could really have been due to arsenic is questionable in the first place; but, in any case, the relation to the chemical form in which arsenic is present and whether it can be bound to protein (as it appears to be in tissues after it is absorbed) are problems well worth further investigation.

DR. CHENOWETH: I would just like to point out that pharmacologists hate nothing more than an impure compound, the impurity of which turns out to be the active material.

DR. H. ZUSSMAN (Geigy Industrial Chemistry Division): The carcinogenicity of iron chelates has perhaps been overrated. Haddow was unable to produce sarcomas with Ferrocene, iron EDTA, iron gluconate and related compounds under the very same conditions conducive to carcinogenesis with iron dextran in mice; there is, indeed, some skepticism that iron dextran is carcinogenic in humans. The Cancer Chemotherapy National Service Center of the National Institutes of Health has reported (unpublished) some degree of anticarcinogenic properties for chelating agents, including diethylenetriaminepentaacetic acid (DTPA).

Long range feeding studies on disodium EDTA in rats were carried out by Dr. Carl Fellers and some of his graduate students at the University of Massachusetts, 1949–1952, supported by Geigy. Regrettably, this work has never been published but the data are available in the Ph.D. theses of J. K. Krum (1948) and S. Yang (1952) in the university library; copies were filed also with the FDA. Brief reference to these studies appeared in Krum, J. K. and C. R. Fellers. *Food Technology* 6: 105, 1952. Investigated in these two-year feeding studies were the effects of marginal as well as high mineral diets, and the effects on reproduction, calcium content of tissues, and dental caries. The studies also included metabolic balance and histology. Concentrations of Na_2EDTA up to 5 per cent in the daily diet were used. It might be remembered that much of this work was done before the development of the more sophisticated attitudes on chelation in biological systems. The results obtained generally agree with those described by Dr. Oser.

Several references to FDA clearance of $CaNa_2EDTA$ in mayonnaise should be clarified. The FDA has recently set a tolerance for it in nonstandardized salad dressings. "Nonstandardized" is a legal term; it does not, however, include mayonnaise. Consequently, at this time the use of EDTA in mayonnaise is still prohibited.

DR. HERTA SPENCER (Montefiore Hospital, New York): This is just a remark in connection with what Dr. Schwarz asked. The remarkable lack of any adverse effect of orally administered EDTA over such a long period of time very well may be due to the poor absorption of EDTA from the gastrointestinal tract. In studies carried out in man under controlled dietary conditions by the balance technique as well as with radioactive calcium, we found that calcium EDTA and the sodium salts of EDTA are very poorly absorbed, perhaps to the extent of 5 to 10 per cent of the administered dose (Laszlo, D. and H. Spencer. *Proceedings of the Conference on Experimental and Clinical Approaches to the Treatment of Poisoning by Radioactive Substances*. Argonne National Laboratory, Chicago, Oct. 5, 1955, ANL-5584, p. 53). These results have recently been confirmed in studies carried out with C^{14}-EDTA (Spencer, H. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 104).

DR. SCHUBERT: I have a question of Dr. Oser. In nonmammalian organisms mutagenic actions of EDTA have been observed. I was wondering whether the offspring of animals given EDTA chronically have been studied from the genetic standpoint?

DR. OSER: No, all litters were carefully examined at birth and at weaning and no signs of malformations were observed. In fact, the pups appeared to be completely normal in all respects.

DR. MALMSTRÖM: It was very interesting, Dr. Westerfeld, to get a summary of quite a number of the properties of the known metalloenzymes. However, I believe that your tables include several debatable points, but I will mention only one of them specifically. In giving the valence states of copper in proteins, you listed hemocyanin as having Cu^{++}. I think a good deal of evidence, for example, magnetic susceptibility (Rawlinson, W. A. *Australian J. Exper. Biol. & Med. Sci.* 18: 185, 1940) and the type of spectral data discussed earlier by Dr. Williams, point to Cu^{+}.

DR. WESTERFELD: This was a paper by Klotz and Klotz (Klotz, I. M. and T. A. Klotz. *Science* 121: 477, 1955).

DR. MALMSTRÖM: The Klotz paper was, however, severely criticized by Dr. Williams (Williams, R. J. P. *Science* 122: 558, 1955).

DR. WILLIAMS: I would like to ask quite a simple question about this protection experiment. Can you tell me if the solutions you are using are free of copper?

DR. WESTERFELD: The enzyme is free of copper. The crucial point is whether we are dealing with the removal of an extraneous metal, such as copper or zinc, which might have gotten into the system to inhibit the enzyme. This cannot be the explanation because the effect of these metal-binding reagents is relatively specific for the cytochrome reduction; we see no effect of these agents on oxygen reduction or methylene blue reduction. The two reactions which are normally

very slowly catalyzed by this enzyme, cytochrome and nitrate reduction, are speeded by these agents, but the reactions which are normally rapidly catalyzed are completely unaffected. So it is not a general activation by removal of extraneous metals.

DR. SCHEINBERG: One point in reference to what Dr. Williams implied. We know that with ceruloplasmin one must remove free ionic copper (that is, copper that is not bound tightly to ceruloplasmin) to avoid pitfalls in studying its enzymatic properties. It is stated in the literature, for example, that ceruloplasmin is a mammalian ascorbic acid oxidase (Holmberg, C. G. and C. B. Laurell. *Acta chem. scand.* 5: 476, 1951). In fact, if one frees a solution of ceruloplasmin of ionic copper by passing it over an ion exchange resin such as IR-120, and then measures the capacity of this ceruloplasmin to catalyze the oxidation of ascorbic acid, one finds that there is no such activity. The ascorbic acid activity of ceruloplasmin solutions is due to *free* copper ions, not to the ceruloplasmin.

DR. PAUL SALTMAN (University of Southern California, Los Angeles): Just one point concerning the nondializability of iron from your transferrin. This is exactly the technique that both Dr. Rubin's group and our own use to measure the binding constants of the protein-metal complex. You just happened to pick a poor chelator in *o*-phenanthroline. EDDHA, EDTA, or citrate will pull the iron completely off of the protein if they are present at a suitable concentration, even at physiological pH. These equilibria are established within 24 hours.

DR. WESTERFELD: I think it is obvious that the total amount of material that could have been included is so massive that I couldn't begin to break it down into each specific case. Neither *o*-phenanthroline nor bipyridyl remove much iron from transferrin even in the presence of reducing agents. Other metal-binding agents might act quite differently. It should also be noted that EDTA does not remove much iron *in vivo* even though it has a high affinity for ferric iron.

DR. DAN A. RICHERT (State University of New York, Syracuse): Does inhibition of an enzyme activity by chelators imply the presence of a metal in that enzyme?

DR. WESTERFELD: Not necessarily. Just because a reagent has metal-binding properties does not preclude the presence of other characteristics which might affect an enzyme.

Summary remarks by the chairman

MAYNARD B. CHENOWETH

The previous preceedings remind me of an oyster with its dainty interior and rugged exterior, for we have ranged from the tasty speculations of the theorists to the hard and practical constructions of the pragmatists. But, then, do not ivory towers rest best upon reinforced concrete?

My complaint that "you cannot prove it" has been countered by the indication that "you can use it." If the chelating agents presently available don't do what is desired, a careful reexamination of the papers by Albert, Foye, and Rubin should provide ample suggestions for further synthesis. On the line of new compounds, one might turn the clock back and ask what became of BAL glucoside. My recollection is that it was too unstable for military purposes, but this might not be a problem now and the material was certainly an impressively effective heavy metal antidote.

My overall impression from the presentation of Westerfeld is that enzymes are remarkably better chelating agents than most of the synthetic molecules so far at hand. Perhaps in light of this compilation of data it is less surprising to find that a powerful chelating agent, fed over a long period of time to animals had relatively little effect upon them. The elaborate studies reported by Oser indicate that we have little to fear from judicious employment of EDTA in our national diet. The rate at which chelating agents will be brought forth for this purpose is sure to be slow, in view of the work involved.

The concept of metal coordination as a mechanism of action of therapeutic and toxic substances is now sufficiently well established to take its place along with the concept of structural analogues as one of the few mechanisms of drug action about which we may say we know something. I hope we are entering upon the logarithmic growth phase and not leaving it.

Consider for a moment the practical problems of assembling the minutes to this lively conference. In this session alone, well over 500 literature references were cited and much original new data presented. If the final printed version of your statements does not sound like you, be grateful. If we did not guess your meaning or misinterpreted, or miscopied, or just plain mistook your thought, let me cite one more reference, Matthew 7:3.

IV. APPLICATIONS OF CHELATING AGENTS IN MEDICINE

HARRY FOREMAN, *Chairman*

Copper metabolism[1]

I. HERBERT SCHEINBERG

Department of Medicine, Albert Einstein College of Medicine and Bronx Municipal Hospital Center, New York City

HUMAN BEINGS REQUIRE COPPER for certain biochemical processes. Yet an excessive amount of copper can be toxic. Unless the amount of copper ingested and absorbed is just sufficient for the body's needs, one of two kinds of mechanisms must exist. If dietary copper is relatively scarce, there must be conservation of copper to avoid the effects of deficiency. If, on the other hand, dietary copper is relatively abundant, there must be limitation of absorption or facilitation of excretion to prevent the excess of copper from producing toxicity. We shall, in this paper, summarize the evidence which indicates that copper is an essential element for human metabolism and the evidence which indicates that it can also be a toxic substance. Then, we shall consider data which suggest that the relation between the body's supply of, and demand for, copper is such that toxicity—not deficiency—is the danger with which the body must cope (92).

The evidence that copper is essential to human metabolism comes from two sources. First, deficiency of copper in several species of animals produces biochemical, pathological, and clinical disturbances. Second, at least seven unique copper proteins have been isolated from human tissues which appear to have specific chemical and physiologic properties (Table 1).

Effects of deficiency of copper have been observed in cattle (83), chickens (35), dogs (101), pigs (13, 20, 34, 41, 58), rats (31, 70) and sheep (48, 68, 99), although not all effects have been observed in each of these. In cattle and sheep natural deficiency occurs generally as the result of an insufficient amount of copper in the forage. It may, however, occur in the presence of an adequate amount of copper either because all or a part of the copper is, in some ill-understood way, "unavailable" (73), or because of the presence in the diet of significant amounts of molybdenum which makes the effects of copper deficiency more severe (25, 30). In chickens (35), dogs (6), pigs (17, 34) and rats (21, 37), the effects of experimental deficiency of copper have been studied chemically, clinically and pathologically. From such investigations of both natural and experimental deficiency, disturbances in at least five biochemical areas have been observed.

1) Absorption and utilization of iron no longer occur normally in copper-deficient pigs (42) or rats (20). The amount of iron absorbed from the gastrointestinal tract has been shown to be diminished by studies with radioactive iron, and iron deficiency eventually ensues. Anemia and a decrease in cytochrome oxidase, which contains heme, iron and copper, are the chief observable effects of the improper metabolism of iron in copper-deficient animals (5, 36, 41, 48). Even parenterally administered iron will not be utilized properly while copper deficiency persists despite the fact that the total turnover of iron can be five times as great as in a normal animal (13). The administration of copper to these animals results in correction of these abnormalities (17).

2) A defect in phospholipid synthesis has been observed in copper-deficient rats. An abnormally low capacity to couple coenzyme A-activated fatty acids to glycerophosphate may be the basis for this disturbance (37).

3) In chickens (35), dogs (6) and pigs (34), a defect in osteoblastic activity, closely resembling that seen in scurvy, has been described.

4) Accompanying the naturally occurring copper deficiency of sheep is an abnormality in wool which has been related to a defect in the cross-linkages of keratin which normally occur through disulfide bridges (99).

5) In copper-deficient rats and sheep, achromotrichia has been observed (35, 99). This may be due to a lowered concentration of the copper-containing protein tyrosinase.

The principal clinical and pathologic effects of copper deficiency in animals are *1*) anemia, which may be indistinguishable from that of iron deficiency or may

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be normochromic and normocytic (16, 17, 101); 2) a demyelinating disease, the causes of which may lie both in the abnormal phospholipid metabolism and the diminution of cytochrome oxidase activity (37, 48); and 3) disturbances in fertility which have been noted in rats (31).

None of the biochemical, clinical or pathologic abnormalities described in copper-deficient animals has ever been noted in human beings (40, 92). Although it is true that some conditions can produce a decrease in the concentration of copper in the serum (15, 16, 57, 79, 92, 94, 98, 112), decreases in total body copper or clinical abnormalities related only to copper deficiency have not been reported. Furthermore, Wilson and Lahey could not induce signs of copper deficiency in 60 days in premature infants to whom they fed a diet containing only 15 micrograms of copper per kilogram of body weight (109).

Despite the absence of demonstrable copper deficiency in human beings, the existence in them of unique copper proteins constitutes additional presumptive evidence that copper is essential to human metabolism. But a word of caution is relevant to a discussion of copper proteins. Because of the widespread distribution of copper and the small amount of the metal present in copper proteins, it has not been easy to be certain whether a protein is a copper protein or whether it merely has been contaminated by copper. For example, both butyryl coenzyme A dehydrogenase (63), and delta amino levulinic acid dehydrase (52) were at first thought to be copper proteins, but subsequent work showed that each merely

TABLE 1. *Characteristics of Human Copper Proteins*

Protein	Source	Mol. Wt.	Sedimentation Constant, $S_{20,w} \times 10^{-13}$	Diffusion Constant, $D_{20,w} \times 10^{-7}$ cm²/sec	Isoelectric Point	Electrophoretic Mobility			Copper Content, %	Color	Absorption Spectrum		Enzymatic Activity*	Copper Removed From Protein by			Reversibility of Copper-Protein Bond*	Immunochemical Specificity*	Physiologic Role	References
						μ cm²/volt-sec. $\times 10^{-5}$	pH	$t_{1/2}$			$E^{1\%}_{1\ cm}$	λ		pH‡	Cyanide‡	DTC†				
Cerebrocuprein I	Human brain	30,000–40,000	3.1	—	—	α_2-β globulin			0.29	Bluish-green	0.14	665	ND	<2.0	—	<5%	ND	ND	Unknown	77
Ceruloplasmin	Human plasma	151,000	7.29	4.5	4.4	−3.46	7.0	0.2	0.34	Blue	9.7 0.68	270 605	D	<3.0	Yes	No	D	D	Unknown	45, 61, 85
Cytochrome c oxidase	Beef heart	93,000	—	—	—	−5.32	8.6	0.1	0.07	—	17	280	D	—	Yes	—	ND	ND	Aerobic oxidation	3, 86
Erythrocuprein	Human red blood cells	28,000 ±2,000	3.02	9.3 ±0.7	5.3	—	—	—	0.32–0.36	Light green-blue	0.10 6.6	655 265	ND	<1.0	Yes	No	ND	D	Unknown	56, 66
Hemocuprein	Ox and human red blood cells and serum	35,000	—	—	—	—	—	—	0.34	Blue	—	—	ND	<1.0	—	No	ND	ND	Unknown	65
Copper protein of liver (Cu LP)	Liver	Approx. 10,000	—	—	—	—	—	—	0.1–2.7	Colorless	7.2 (0.1% Cu) 3.0 (2.7% Cu)	270–275 (peak) 255–265 (inflexion)	ND	—	—	Yes (>60 min)	D	ND	Unknown	72
Tyrosinase	Mouse melanoma	—	—	—	—	—	—	—	0.22–0.25	—	—	—	D	—	Yes	—	D	ND	Production of melanin	11, 12, 62

* D = Demonstrated; ND = Not Demonstrated. † DTC = Diethyldithiocarbamate. The reaction of protein-bound copper with this compound is so complex that the various studies are not strictly comparable. For example, the time over which the reaction is observed, the pH of the solution and the presence of other proteins can affect the reaction sufficiently so that its strict characterization can be given only if the conditions of the reaction are precisely defined. ‡ To a lesser extent the same considerations apply to the reaction of protein-bound copper with H^+ and CN^- as with DTC.

had been contaminated with the metal (96, 110). Table 1 lists seven proteins which *1*) contain copper as an integral part of the molecule, *2*) are unique, and *3*) are present in human beings. The essentiality of copper to the integrity of these proteins has been shown by demonstrating that the copper is bound very tightly to the molecule (in that it is undialyzable, passes through ion-exchange columns, and reacts slowly if at all with copper-complexing reagents) or by showing that some other characteristic of the protein is dependent on the presence of copper in the molecule. The uniqueness of each of these proteins is a consequence of their specific chemical, immunochemical, physiologic, or genetic properties.

The copper-protein bond has been shown to be very tight in four of these compounds by the fact that it is not broken rapidly by diethyldithiocarbamate (DTC) or by a concentration of hydrogen ions as high as 10^{-4} molar. Copper which has merely been added to proteins, in contrast, is generally readily freed and rendered dialyzable, by either DTC or a pH of 4.0. Furthermore, in five of the seven proteins either the absorption spectrum is modified or the enzymatic activity is abolished, or both occur, if copper is removed from the molecule by DTC, hydrogen ions or cyanide ions: cerebrocuprein loses its light absorption maximum at 665 mμ (76, 77); ceruloplasmin loses its maximum at 605 mμ and its oxidase activity (45, 61); cytochrome c oxidase loses its characteristic absorption at 445 mμ and its enzymatic activity (104); the liver copper protein, Cu LP, loses its ultraviolet absorption at 255–265 mμ (72); and tyrosinase loses its oxidase activity (62). In addition, if the copper is removed under certain conditions from ceruloplasmin (26, 70), Cu LP (72), or tyrosinase (62), each apoprotein can re-combine specifically with copper to regenerate the native protein without essential change.

The differing characteristics of these proteins given in Table 1 strongly suggest that each is unique. Confirmation of this is found in the cases of ceruloplasmin and tyrosinase since there are human subjects who have inherited, in autosomal recessive fashion, marked deficiency or absence of either one of these proteins but not of both. Nor do individuals who are deficient in either ceruloplasmin or tyrosinase give indication of deficiency of any other copper protein. One gene directs the synthesis of one protein, or even of one polypeptide chain of a protein, according to current genetic concepts (43, 51). Consequently, it is virtually certain, from this genetic evidence, that ceruloplasmin and tyrosinase are different proteins, and it is very likely that each of them also differs from the other copper proteins of Table 1.

A priori it seems highly unlikely that these specific proteins, each requiring copper as an essential part of its structure, would exist in man if they had no function. Together with the manifestations of copper deficiency in animals they are the reasons for stating that copper is essential to human metabolism.

Some substances, such as vitamin D, which are essential to health can be toxic in excessive amounts. This is true of copper. Chronic copper toxicity occurs naturally in sheep as a result of which anemia, with or without hemolytic crises, and marked liver disease develop (2, 68, 99). In chickens hemolytic anemia has been produced experimentally by copper (39). In fish kept in water containing 1 microgram of copper per milliliter, evidence of renal and neural degeneration has been found (102). Histologic manifestations of experimental copper poisoning have been seen in mice (103) and rats (49, 111).

In human beings, acute and chronic copper toxicity occur but each is uncommon (28). Acute toxicity probably is averted by the vomiting and diarrhea which usually follow the ingestion of more than 10 to 15 mg of copper in one dose and which leave little copper available for absorption. One man, who apparently ingested 20 gm of copper sulfate at one time and had been doing so several times weekly for months, was admitted to a hospital with hemolytic anemia, but such a case must be extraordinarily rare (80).

We believe chronic copper toxicity in human beings is synonymous with hereditary hepatolenticular degeneration, or Wilson's disease. Almost all of the tissues of individuals with this illness have higher concentrations of copper than normal, and in some instances almost a hundred-fold higher (9, 18, 24, 40, 90). Furthermore, it recently has become increasingly clear that a marked elevation in the concentration of copper precedes the development of the histologic and physiologic damage in the liver, which ultimately may be fatal (4, 90). Finally, if the intake and absorption of dietary copper in these patients are reduced, and the urinary excretion of copper is promoted—for which there are available pharmacologic means—there is a definite and sustained improvement in the neurologic symptoms and signs of many patients (23, 29, 60, 74, 91, 93, 105, 107). Improvement in hepatic disease has not, however, been notable in most patients, and it is still too early to know whether such an "anti-copper" regimen can prevent the development of Wilson's disease in those asymptomatic subjects in whom this hereditary illness is virtually certain to develop. Nevertheless, it is difficult to believe, from the evidence already at hand, that chronic copper toxicity is not the basis of this disease.

Copper, then, is essential to human metabolism, and an excess of it can be fatally toxic. Yet deficiency and toxicity of copper are both highly unusual occurrences in man. This could, as we have indicated, be the happy result of the presence, in all human diets, of just enough copper to supply the body's needs. But such a fortunate coincidence is not the case, as Table 2 shows.

If we make the reasonable assumption that the body content of each metal is an index of the respective requirement for copper, or for iron, with which we shall find comparison useful, we see in Table 2 that the body has about 40 times as much iron as copper. But an average diet contains only 4 times as much iron as copper. Consequently, relative to need, there is about 10 times as much copper as iron in our food. In addition,

TABLE 2. *Dietary Supply, Body Stores and Loss With Blood of Iron and Copper in a 70 kg Man*

	Daily Dietary Supply, mg	Total Amount in Body, mg	% of Body Content Lost with 100 ml Blood	References
Iron	12–15	4000–5000	1.0–1.25	99
Copper	2–5	75–150	0.067–0.13	40

significant amounts of iron can be lost by bleeding (because of the amount of iron in hemoglobin, of course) but the loss of copper with blood is negligible. It seems clear, therefore, why copper deficiency does not occur in human beings since even iron deficiency is rare except in the presence of growth, pregnancy or bleeding.

What about toxicity? In a year the diet supplies about ten times as much copper as the total in the body. And we take in an unknown additional amount from copper plumbing, kitchen utensils, beer-brewing kettles and whiskey stills (22). To avoid poisoning, we must excrete practically all we ingest through the feces (copper is not excreted appreciably *via* any other route) either by absorbing practically none of it, or by excreting into the gut that which we do absorb. Whether one or both of these occurs is uncertain since studies with labeled copper, necessary to decide this issue, cannot be carried out for longer than a few days because Cu^{64}, the generally available isotope, has a half-life of only 12.8 hours (108).

Nevertheless, without clearly defining it, the results of several authors (8, 14, 53, 69) have indicated that some mechanism exists which rids the body of the excess of copper ingested. Thus, with ingestion of 1 to 12.5 mg of Cu^{64}, from 95 per cent of the smaller (14) to 26 per cent of the larger dose (8) was recovered in the feces, over a period of more than four days. In all of these experiments an appreciable amount of copper, and almost certainly an amount greater than the body needs, probably was absorbed from the gut and presumably subsequently excreted into it.

Studies in which Cu^{64} was administered intravenously have supported this conclusion. In dogs 7 per cent of intravenously administered copper was excreted and, of this, 5.7 and 0.9 per cent were excreted in the bile and *via* the intestinal wall, respectively, with 0.4 per cent appearing in the urine (64). In human studies, up to 33 per cent of intravenously administered Cu^{64} was recovered in the feces (8) and a considerable porton of this reached the intestinal lumen *via* the bile.

It appears, then, that the body has at least one mechanism for fecal excretion of dietary copper which seems to prevent the relatively great excess of dietary copper from becoming toxic. Some further insight into the nature of this regulation can be obtained by returning to a consideration of those human beings in whom chronic copper toxicity develops, that is, patients with Wilson's disease.

There are four reasons which make it probable that the copper protein, ceruloplasmin, is essential to this mechanism. First, deficiency, or even absence, of ceruloplasmin occurs in almost all patients with Wilson's disease (7, 9, 18, 89, 106). With rare exceptions (19, 33, 107), they have 0–15 mg of ceruloplasmin per 100 ml of plasma, while normal subjects generally possess 20 to 35 mg per 100 ml. Second, deficiency of ceruloplasmin is probably life-long in these patients and has been found to have antedated symptoms or signs of Wilson's disease in at least seven children (38, 60, 93). Third, such asymptomatic and hypoceruloplasminemic children show no other clinical abnormality to indicate any other physiologic role for ceruloplasmin. Fourth, we have already referred to the generally accepted thesis of modern genetics that the function of one gene is to direct the synthesis of one protein (43, 51) so that, having found ceruloplasmin synthesis to be defective in this autosomal and recessively inherited disease, it is very unlikely that a second protein abnormality is present, unless this protein is a precursor, or product of ceruloplasmin or an enzyme essential for the synthesis of the latter. Indeed, we have already indicated that there is evidence that even the other cooper proteins are not deficient in individuals with Wilson's disease, since tyrosinase, erythrocuprein (Cartwright, G. E., personal communication), cerebrocuprein (75) and the liver copper protein, Cu LP (72) have been shown to be present normally in one or more of these patients.

Enough facts are available to compare and contrast normal balanced copper metabolism with the unbalanced metabolism characteristic of Wilson's disease (Fig. 1). After intestinal absorption much of the copper is deposited in the liver to be incorporated, in minutes to hours, into the liver copper protein, Cu LP. There copper quite probably is bound tightly to sulfhydryl groups since it requires an agent possessing sulfhydryl groups, like DTC, or an agent with a higher affinity for sulfhydryl groups than copper, like *p*-mercuribenzoate, to displace the metal from Cu LP. To this point in both normal subjects and in patients with Wilson's disease the observed aspects of copper metabolism appear to be identical, including the hepatic concentration of Cu LP and all of the latter's characteristics except copper content.

What now occurs in normal individuals? In serial observations in rabbits, to which Cu^{64} is administered, the specific activity of copper in ceruloplasmin approaches, but does not exceed, its specific activity in Cu LP within hours (Morell, A. G., unpublished observations). In normal human subjects, orally administered Cu^{64} soon appears in the blood as free copper in high concentration which rapidly falls. Then Cu^{64} reappears in blood as part of the ceruloplasmin molecule. From both of these observations we conclude that a considerable but uncertain fraction of Cu LP copper is transferred in the normal individual to ceruloplasmin as part of which it circulates in plasma. Bound to a molecule of 151,000 molecular weight, little copper diffuses through capillary membranes to intercellular or intracellular compartments. Eventually, either in the liver or in the intestinal wall, copper is liberated from ceruloplasmin and excreted in the feces.

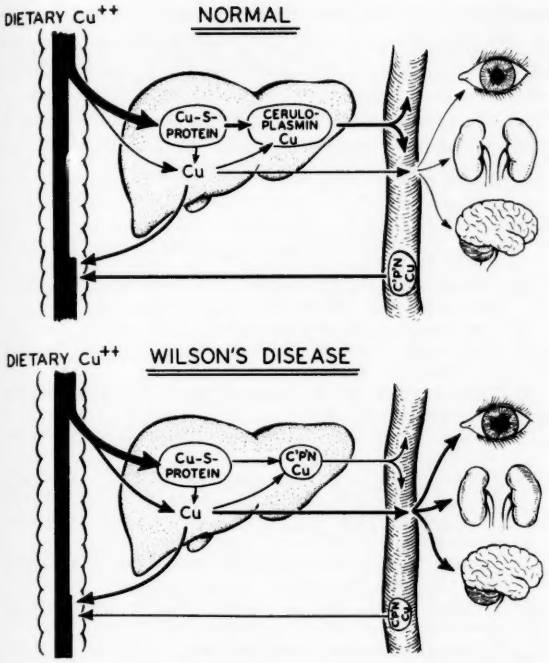

FIG 1. Copper metabolism in normal subjects and patients with Wilson's disease.

In the patient with Wilson's disease, the transfer of copper from Cu LP to ceruloplasmin does not take place. The exact nature of this block is unknown but it would be a reasonable result of a genetic defect in the synthesis of ceruloplasmin. A portion of this hepatic copper reaches the plasma in time but, unbound to ceruloplasmin, it is relatively free to diffuse through capillary membranes to inter- and intracellular compartments as well as into the feces, urine, cerebrospinal fluid and other secretions. Much of it certainly is excreted, but more of it penetrates cells than in the normal. Although this amount may be only slightly greater than that entering the cells of normal individuals, after years a great excess of tissue copper easily could occur and produce, presumably, the pathology which characterizes Wilson's disease.

Apart from whether this theoretical formulation carries conviction, four facts are difficult to reconcile with such a central role for ceruloplasmin in regulating copper metabolism. First, the age at onset and severity of Wilson's disease in a patient are not well correlated with his serum concentration of ceruloplasmin (19). Second, subjects with several other conditions can be deficient in serum ceruloplasmin (92). Third, several patients with this disease, on one or more occasions, have been shown to possess a normal concentration of ceruloplasmin (33, 81, 87, 107) and there is an additional small number of patients whose concentration of this protein is only slightly lower than normal (19). Fourth, parents of patients with Wilson's disease, who are heterozygous for its abnormal gene and in whom the disease has never been seen to develop, may, rarely, exhibit concentrations of ceruloplasmin (as low as 9 mg per 100 ml) which are indistinguishable from the low concentrations characteristic of patients (9, 19, 95).

Age at onset and severity of the disease may be dependent on the intake of dietary copper as well as on the concentration of ceruloplasmin. Daily diets with as little as two or more than 10 milligrams of copper are not unusual. And, in all of the other conditions where ceruloplasmin deficiency occurs, it is transient, in comparison with the minimum of seven to eight years required for Wilson's disease to manifest itself.

Certainly patients with indubitable Wilson's disease and normal concentrations of ceruloplasmin and healthy heterozygotes with low concentrations of ceruloplasmin would not seem compatible with the hypothesis presented. However, two explanations could account for patients with normal concentrations of the protein, the second of which might apply also to the healthy heterozygote with low concentrations. First, an increase in estrogens, whether exogenous or endogenous, as in pregnancy, can elevate the serum concentration of ceruloplasmin in normal individuals and in some patients with Wilson's disease (1, 7, 19, 32, 44, 50, 54, 59, 67, 82, 84, 97). Since hepatic decompensation can increase estrogen concentration, and occurs frequently in the later stages of Wilson's disease, a normal concentration of ceruloplasmin in a patient may be a result of hepatic insufficiency (33, 87). We have seen one patient whose characteristically low concentration of the protein changed to a normal value at a time when the function of his liver was considerably impaired (Sternlieb, I., I. H. Scheinberg, and J. Kurtzke, unpublished observations).

Second, ceruloplasmin, like transferrin, haptoglobin, albumin and hemoglobin, is heterogeneous (10, 27, 71, 100). In human beings several molecular variants of ceruloplasmin have been described, identical with respect to such characteristic properties as absorption spectrum, oxidase activity and copper content, but distinguishable by electrophoretic and chromatographic behavior. It is possible that some variants of ceruloplasmin exist which possess characteristics by which the concentration of the protein is measured—blue color, immunochemical specificity or oxidase activity, for examples—but which lack whatever molecular feature is responsible for the physiologic role of ceruloplasmin. A somewhat, but not entirely, similar explanation recently has been proposed to account for the deficiency of hemoglobin in thalassemia (51). Inconclusive evidence has also been found in one patient with Wilson's disease and a normal concentration of ceruloplasmin that the latter may have been an abnormal variant, at least electrophoretically (Morell, A. G., I. H. Scheinberg, and A. Sass-Kortsak, unpublished observations). This explanation perhaps also could account for why heterozygotes remain healthy even in the face of a low concentration of the protein since part of their ceruloplasmin is normal qualitatively whereas the patient with the same level may have only an abnormal variant of ceruloplasmin.

SUMMARY

Copper is essential in several areas of human metabolism, yet the deposition of excess amounts of copper in tissues can be toxic. The dietary supply of copper, in almost all parts of the world, is so great, relative to metabolic requirements, that human deficiency of copper rarely, if ever, occurs. The potential toxicity of copper is the danger with which the body must cope. Studies of human subjects who probably have an hereditary inability to prevent toxicity of dietary copper indicate that the plasma copper protein, ceruloplasmin, plays a central role in regulating copper metabolism and preventing its toxicity in normal individuals.

REFERENCES

1. Adelstein, S. J., T. L. Coomes and B. L. Vallee. *New England J. Med.* 255: 105, 1956.
2. Albiston, H. E., L. B. Bull, A. T. Dick and J. C. Keast. *Australian Vet. J.* 16: 233, 1940.
3. Ambe, K. S. and A. Venkataraman. *Biochem. Biophys. Res. Comm.* 1: 133, 1959.
4. Anderson, P. J. and H. Popper. *Am. J. Path.* 36: 483, 1960.
5. Anderson, R. L. and S. B. Tove. *Nature, London* 182: 315, 1958.
6. Baxter, J. H. and J. J. Van Wyk. *Bull. Johns Hopkins Hosp.* 93: 1, 1953.
7. Bearn, A. G. *Am. J. Med.* 22: 747, 1957.
8. Bearn, A. G. and H. G. Kunkel. *J. Lab. & Clin. Med.* 45: 623, 1955.
9. Bickel, H., F. C. Neale and G. Hall. *Q. J. Med.* 26: 527, 1957.
10. Broman, L. *Nature, London* 182: 1655, 1958.
11. Brown, F. C. and D. N. Ward. *Proc. Soc. Exper. Biol. & Med.* 100: 701, 1959.
12. Brown, F. C., D. N. Ward and A. C. Griffin. In: *Pigment Cell Biology*, edited by M. Gordon. New York: Acad. Press, 1959, p. 525.
13. Bush, J. A., W. N. Jensen, J. W. Athens, H. Ashenbrucker, G. E. Cartwright and M. M. Wintrobe, *J. Exp. Med.* 103: 701, 1956.
14. Bush, J. A., J. P. Mahoney, H. Markowitz, C. J. Gubler, G. E. Cartwright and M. M. Wintrobe. *J. Clin. Invest.* 34: 1766, 1955.
15. Butterworth, C. E., Jr., C. J. Gubler, G. E. Cartwright and M. M. Wintrobe. *Proc. Soc. Exper. Biol. & Med.* 98: 594, 1958.
16. Cartwright, G. E. *Am. J. Clin. Nutrition* 3: 11, 1955.
17. Cartwright, G. E., C. J. Gubler, J. A. Bush and M. M. Wintrobe. *Blood* 11: 143, 1956.
18. Cartwright, G. E., R. E. Hodges, C. J. Gubler, J. P. Mahoney, K. Daum, M. M. Wintrobe and W. B. Bean. *J. Clin. Invest.* 33: 1487, 1954.
19. Cartwright, G. E., H. Markowitz, G. S. Shields and M. M. Wintrobe. *Am. J. Med.* 28: 555, 1960.
20. Chase, M. S., C. J. Gubler, G. E. Cartwright and M. M. Wintrobe. *J. Biol. Chem.* 199: 757, 1952.
21. Chase, M. S., C. J. Gubler, G. E. Cartwright and M. M. Wintrobe. *Proc. Soc. Exper. Biol. & Med.* 80: 749, 1952.
22. *Copper and Brass Bulletin, No. 189*, New York: Copper and Brass Res. Assoc., 1959.
23. Cumings, J. N. *Proc. Roy. Soc. Med.* 52: 62, 1959.
24. Cumings, J. N. *Heavy Metals and the Brain.* Springfield, Ill.: Thomas, 1959.
25. Cunningham, I. J. In: *A Symposium on Copper Metabolism.* Baltimore: Johns Hopkins Press, 1950, p. 246.
26. Curzon, G. *Proc. Roy. Soc. Med.* 52: 64, 1959.
27. Curzon, G. and L. Vallet. *Biochem. J.* 74: 279, 1960.
28. Davenport, S. J. *Review of literature on health hazards of metals I. Copper.* U. S. Dept. of the Interior, Information Circular No. 7666, 1953.
29. Denny-Brown, D. and H. Porter. *New England J. Med.* 245: 917, 1951.
30. Dick, A. T. *Nature, London* 172: 637, 1953.
31. Dutt, B. and C. F. Mills. *J. Comp. Path.* 70: 120, 1960.
32. Elsner, P. and O. Hornykiewicz. *Arch. Gynaek.* 185: 251, 1954.
33. Enger, E. *Acta med. scandinav.* 163: 121, 1959.
34. Follis, R. H., Jr., J. A. Bush, G. E. Cartwright and M. M. Wintrobe. *Bull. Johns Hopkins Hosp.* 97: 405, 1955.
35. Gallagher, C. H. *Australian Vet. J.* 33: 311, 1957.
36. Gallagher, C. H., J. D. Judah and K. R. Rees. *Proc. Roy. Soc. Med.* 145: 134, 1956.
37. Gallagher, C. H., J. D. Judah and K. R. Rees. *Proc. Roy. Soc. Med.* 145: 195, 1956.
38. Gastager, H. and H. Tschabitscher. *Munchen. med. Wchnschr.* 100: 1961, 1958.
39. Goldberg, A., C. B. Williams, R. S. Jones, M. Yanagita, G. E. Cartwright and M. M. Wintrobe. *J. Lab. & Clin. Med.* 48: 422, 1956.
40. Gubler, C. J. *J.A.M.A.* 161: 530, 1956.
41. Gubler, C. J., G. E. Cartwright and M. M. Wintrobe. *J. Biol. Chem.* 224: 533, 1957.
42. Gubler, C. J., M. E. Lahey, M. S. Chase, G. E. Cartwright and M. M. Wintrobe. *Blood* 7: 1075, 1952.
43. Harris, H. *Human Biochemical Genetics.* Cambridge Univ. Press, 1959.
44. Holmberg, C. G. and C. B. Laurell. *Acta Chem. Scandinav.* 1: 944, 1947.
45. Holmberg, C. G. and C. B. Laurell. *Acta Chem. Scandinav.* 2: 550, 1948.
46. Holmberg, C. G. and C. B. Laurell. *Acta Chem. Scandinav.* 5: 476, 1951.
47. Holmberg, C. G. and C. B. Laurell. *Acta Chem. Scandinav.* 5: 921, 1951.
48. Howell, J. McC. and A. N. Davison. *Biochem. J.* 72: 365, 1959.
49. Howell, J. S. *J. Path. Bact.* 77: 473, 1959.
50. Humoller, F. L., M. P. Mockler, J. M. Holthaus and D. J. Mahler. *J. Lab. & Clin. Med.* 56: 222, 1960.
51. Ingram, V. M. and A. O. W. Stretton. *Nature, London* 184: 1903, 1959.
52. Iodice, A. A., D. A. Richert and M. P. Schulman. *Fed. Proc.* 17: 248, 1958.
53. Jensen, W. H. and H. Kamin. *J. Lab. & Clin. Med.* 49: 200, 1957.
54. Johnson, N. C., T. Kheim and W. B. Kountz. *Proc. Soc. Exper. Biol. & Med.* 102: 98, 1959.
55. Keltz, A. *Fed. Proc.* 18: 258, 1959.
56. Kimmel, J. R., H. Markowitz and D. M. Brown. *J. Biol. Chem.* 234: 46, 1959.
57. Lahey, M. E., M. Behar, F. Viteri and N. S. Scrimshaw. *Pediatrics* 22: 72, 1958.
58. Lahey, M. E., C. J. Gubler, M. S. Chase, G. E. Cartwright and M. M. Wintrobe. *Blood* 7: 1053, 1952.
59. Lahey, M. E., C. J. Gubler, G. E. Cartwright and M. M. Wintrobe. *J. Clin. Invest.* 32: 329, 1953.
60. Lange, J. and H. Hager. *Ztschr. Kinderh.* 84: 125, 1960.
61. Laurell, C. B. In: *The Plasma Proteins*, edited by F. W. Putnam. New York: Acad. Press, 1960, p. 349.
62. Lerner, A. B. *Arch. Biochem. Biophys.* 36: 473, 1952.
63. Mahler, H. R. *J. Biol. Chem.* 206: 13, 1954.
64. Mahoney, J. P., J. A. Bush, C. J. Gubler, W. H. Moretz, G.E. Cartwright and M. M. Wintrobe. *J. Lab. & Clin. Med.* 46: 702, 1955.
65. Mann, T. and D. Keilin. *Proc. Roy. Soc., London., ser. B.* 126: 303, 1938.

66. MARKOWITZ, H., G. E. CARTWRIGHT AND M. M. WINTROBE. *J. Biol. Chem.* 234: 40, 1959.
67. MARKOWITZ, H., C. J. GUBLER, J. P. MAHONEY, G. E. CARTWRIGHT AND M. M. WINTROBE. *J. Clin. Invest.* 34: 1498, 1955.
68. MARSTON, H. R. *Physiol. Rev.* 32: 65, 1952.
69. MATTHEWS, W. B. *J. Neurol. Psychiat.* 17: 242, 1954.
70. MORELL, A. G. AND I. H. SCHEINBERG. *Science* 127: 588, 1958.
71. MORELL, A. G. AND I. H. SCHEINBERG. *Science* 131: 930, 1960.
72. MORELL, A. G., J. R. SHAPIRO AND I. H. SCHEINBERG. *Symposium on Wilson's Disease*, London, September 20–22, 1960.
73. PALSSON, P. A. AND H. GRIMSSON. *Proc. Soc. Exper. Biol. & Med.* 83: 518, 1953.
74. PLOOIJ, M., J. VAN DER MEER AND A. KAAG. *Nederl. tijdschr. geneesk.* 103: 200, 1959.
75. PORTER, H. *A.M.A. Arch. Neurol.* 1: 544, 1959.
76. PORTER, H. AND S. AINSWORTH. *Proc. Soc. Exper. Biol. & Med.* 98: 277, 1958.
77. PORTER, H. AND S. AINSWORTH. *J. Neurochem.* 5: 91, 1959.
78. PORTER, H. AND J. FOLCH. *A.M.A. Arch. Neurol. Psychiat.* 77: 8, 1957.
79. REIFF, B. AND H. SCHNIEDEN. *Blood* 14: 967, 1959.
80. ROBERTS, R. H. *Mississippi Doctor* 33: 292, 1956.
81. ROSENOER, V. M. AND G. FRANGLEN. *Lancet* 2: 1163, 1959.
82. RUSS, E. M., J. RAYMUNT AND S. PILLAR. *J. Clin. Endocrinol.* 17: 908, 1957.
83. RYS, R. *Nature* 183: 1596, 1959.
84. SALA, I. AND L. GAMBARA. *Lattante* 28: 458, 1957.
85. SANDERS, B. E., O. P. MILLER AND M. N. RICHARD. *Arch. Biochem. Biophys.* 84: 60, 1959.
86. SANDS, R. H. AND H. BEINERT. *Biochem. Biophys. Res. Comm.* 1: 175, 1959.
87. SASS-KORTSAK, A., M. CHERNIAK, D. W. GEIGER AND R. J. SLATER. *J. Clin. Invest.* 38: 1672, 1959.
88. SCHEINBERG, I. H. *Progr. Neurobiol.*, 1956, p. 52.
89. SCHEINBERG, I. H. AND D. GITLIN. *Science* 116: 484, 1952.
90. SCHEINBERG, I. H. AND I. STERNLIEB. *Gastroenterology* 37: 550, 1959.
91. SCHEINBERG, I. H. AND I. STERNLIEB. *Am. J. Med.* 29: 316, 1960.
92. SCHEINBERG, I. H. AND I. STERNLIEB. *Pharmacol. Rev.* 12: 355, 1960.
93. SCHEINBERG, I. H. AND I. STERNLIEB. *Ann. Int. Med.* (in press).
94. SCHUBERT, W. K. AND M. E. LAHEY. *Pediatrics* 24: 710, 1959.
95. STERNLIEB, I., A. G. MORELL AND I. H. SCHEINBERG. *Symposium on Wilson's Disease*, London, September 20–22, 1960.
96. STEYN-PARVÉ, E. P. AND H. J. BEINERT. *J. Biol. Chem.* 233: 843, 1958.
97. STUDNITZ, W. V. AND D. BEREZIN. *Acta endocrinol.* 27: 245, 1959.
98. STURGEON, P. AND C. BRUBAKER. *A.M.A. J. Dis. Child.* 92: 254, 1956.
99. UNDERWOOD, E. J. *Trace Elements in Human and Animal Nutrition*. New York: Acad. Press, 1956.
100. URIEL, J., H. GÖTZ AND P. GRABAR. *Schweiz. med. Wchnschr.* 87: 431, 1957.
101. VAN WYK, J. J., J. H. BAXTER, J. H. AKEROYD AND A. G. MOTULSKY. *Bull. Johns Hopkins Hosp.* 93: 41, 1953.
102. VOGEL, F. S. *J. Exper. Med.* 110: 801, 1959.
103. VOGEL, F. S. *Am. J. Path.* 36: 699, 1960.
104. WAINIO, W. W., C. VANDER WENDE AND N. F. SHIMP. *J. Biol. Chem.* 234: 2433, 1959.
105. WALSHE, J. M. *Am. J. Med.* 21: 487, 1956.
106. WALSHE, J. M. *Brit. M. Bull.* 13: 132, 1957.
107. WALSHE, J. M. *Lancet* 1: 188, 1960.
108. WAY, K., R. W. KING, C. L. MCGINNIS AND R. VAN LIESHOUT. *Nuclear level schemes*. A-40-A-92. National Academy of Sciences, National Res. Council, Washington, 1955.
109. WILSON, J. F. AND M. E. LAHEY. *Pediatrics* 25: 40, 1960.
110. WILSON, M. L., A. A. IODICE, M. P. SCHULMAN AND D. A. RICHERT. *Fed. Proc.* 18: 352, 1959.
111. WOLFF, S. M. *A.M.A. Arch. Path.* 69: 217, 1960.
112. ZIPURSKY, A., H. DEMPSEY, H. MARKOWITZ, G. CARTWRIGHT AND M. M. WINTROBE. *A.M.A. J. Dis. Child.* 96: 148, 1958.

DISCUSSION

Biochemical and pharmacological properties of the metal-binding agent penicillamine[1]

H. VASKEN APOSHIAN[2]

Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennesse

THE PURPOSE OF THIS DISCUSSION is to review the biochemical and pharmacological properties of penicillamine, a metal-binding agent which increases the urinary excretion of copper. This drug, at present, is the drug of choice for the oral treatment of Wilson's disease (16–18).

The activities of penicillamine and its derivatives (Fig. 1) in stimulating the urinary excretion of copper (16–18) and in protecting against the lethal effects of mercuric chloride (1–3) are summarized in Table 1. Of these compounds only penicillamine has an effect on both copper and mercuric chloride. It increases the urinary

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[2] National Institutes of Health Senior Research Fellow. Present address: Dept. of Biochemistry, Stanford University School of Medicine, Palo Alto, Calif.

TABLE 1. *Summary of Influence of Penicillamine Analogues on Urinary Excretion of Copper and on Protection of Rats Against Lethal Effects of Mercuric Chloride*

I. *Increase the urinary excretion of copper in Wilson's disease:*
D-penicillamine (16–18)
DL-penicillamine (16–18)
β-methyl-β-ethyl cysteine
II. *Without effect on the urinary excretion of copper in Wilson's disease:*
N-acetyl-DL-penicillamine*
β-methyl cysteine (2)
cysteine (17)
III. *Protection against the lethal effects of mercuric chloride in the rat:*
a) active:
N-acetyl-DL-penicillamine (3)
N-acetyl-β-methyl-β-ethyl cysteine†
D-penicillamine (1, 3)
DL-penicillamine (1, 3)
b) inactive:
L-penicillamine (1, 3)
β-methyl cystine (2)
cysteine (1, 3)

* Personal communication from I. H. Scheinberg. † Unpublished, H. V. Aposhian.

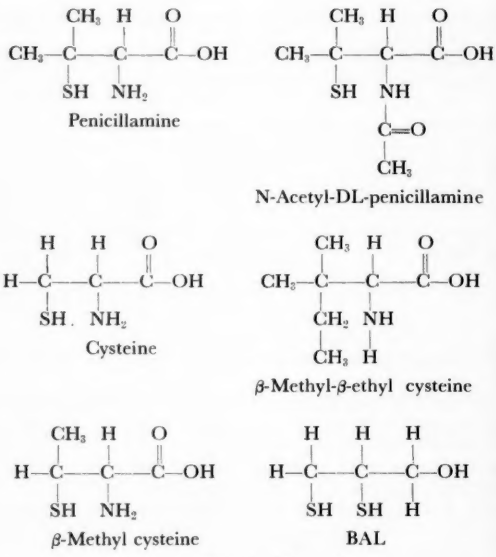


FIG. 1. Chemical formulas.

excretion of copper and protects against mercuric chloride. N-Acetyl-DL-penicillamine does not increase copper excretion but does protect against the lethal effects of mercuric chloride (1, 3). It is not known whether this specificity is due to differences in the chemical affinities of the metals for N-acetyl-DL-penicillamine or due to the metabolic properties of the metals and the sulfhydryl compound. The specificity in action of N-acetyl-DL-penicillamine, even though the studies are limited to two metals, suggests that further simple changes in the chemical structure of penicillamine may increase the specificity of its binding to these and perhaps other metals.

The metal-binding activity of penicillamine is only one of its biological properties. The LD_{50} of a single oral dose of DL-penicillamine for the rat is 365 mg/kg (3). Deaths occur after running fits, shrieking, salivation, and convulsions. However, the oral administration of 1000 mg N-acetyl-DL-penicillamine per kg or the oral administration of 1200 mg D-penicillamine per kg to rats does not result in any observable toxic reaction (3).

The inhibitory activity of L-penicillamine has been demonstrated in a variety of biological systems. L-Penicillamine inhibits the growth of rats receiving a choline-free diet. This inhibition is reversed by either aminoethanol, its N-methyl derivatives, or vitamin B_6 (11, 19). Evidence for the inactivation of pyridoxal-5-phosphate by L-penicillamine via a suggested thiazolidene formation has been presented (10). L-Penicillamine inhibits the growth of *L. mesenteroides* P-60 (7, 8), *S. carlsbergensis* (4, 6), or *E. coli* (4). The branched chain amino acids, isoleucine, valine, or leucine, reverse the inhibitory activity of this sulfhydryl amino acid in *E. coli* (4).

The inhibition by L-penicillamine, and to a lesser extent by D-penicillamine, of liver transaminases (10, 12), liver cysteine desulfhydrase (5) and of the synthesis of γ-aminolaevulic acid (13), each of which appears to require pyridoxal-5-phosphate as the coenzyme, points out the metabolic involvement of L-penicillamine with pyridoxal-5-phosphate and suggests the biochemical basis for the toxicity of L-penicillamine. L-Penicillamine not only increases the urinary excretion of copper (17), but it also increases the urinary excretion of vitamin B_6 (12). The levels of vitamin B_6 in patients with Wilson's disease and the influence of penicillamine on such levels have not been studied. If the beneficial effects of penicillamine in the therapy of Wilson's disease are due solely to its increasing copper excretion, D-penicillamine, not L- or DL-penicillamine, should be the agent of choice, since the inhibitory and toxic properties of the D-isomer are much less than those of the L-isomer (3).

Penicillamine also influences the excretion of iron and lead (9).

What is the explanation for the difference in the effect of penicillamine and its analogues on the excretion of copper? How are these compounds metabolized? Cysteine and the diastereoisomers of β-methyl cysteine are rapidly deaminated by L-amino acid oxidase (Fig. 2). L-Penicillamine is not. Further studies with L-penicillamine and measurement of ammonia production, a product of the reaction catalyzed by L-amino acid oxidase, indicate that L-penicillamine is not oxidatively deaminated by L-amino acid oxidase (5).

When the action of D-amino acid oxidase is studied, the same phenomena are observed. Cysteine and the β-methyl compounds are oxidized at a rapid rate. Although penicillamine is deaminated by D-amino acid oxidase, the rate of oxygen consumption is much slower (Fig. 3) (5).

On the basis of these enzymological experiments, it was predicted that the β-methyl cysteine diastereoisomers would be inactive in the treatment of Wilson's disease

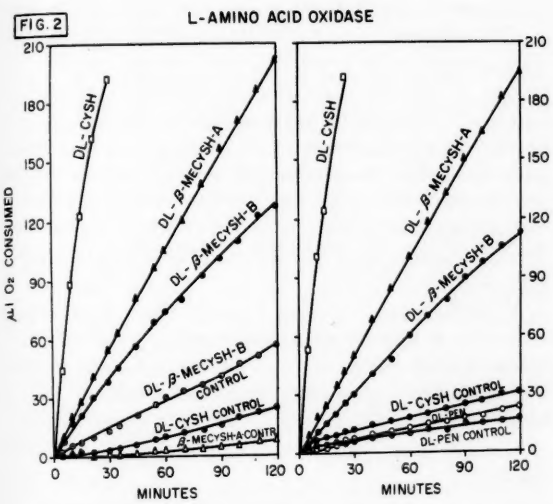


FIG. 2. Metabolism of sulfhydryl amino acids by L-amino acid oxidase. *Agkistrodon p. piscivorus* (cottonmouth) venom used as source of enzyme. Forty micromoles of DL-amino acid were present in a final volume of 3.0 ml. Abbreviations: CySH = cysteine; Pen = penicillamine; β-MeCySH = β-methyl cysteine. Experiments marked *control* were incubated with enzyme omitted (2).

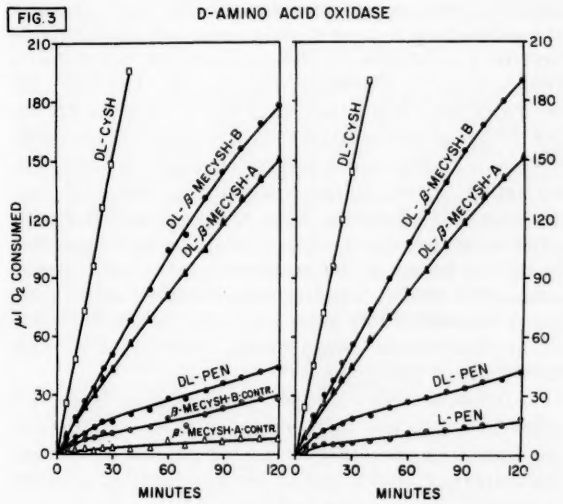


FIG. 3. Metabolism of sulfhydryl amino acids by D-amino acid oxidase. Hog kidney used as source of enzyme. Forty micromoles of DL-amino acid were present in a final volume of 2.5 ml. Abbreviations: see Fig. 2 (2).

or of mercuric chloride intoxication. When tested, they were found to be inactive (2).

Neither enantiomorph of penicillamine nor the β-methyl cysteine compounds are desulfhydrated by rat liver cysteine desulfhydrase (5). In fact, penicillamine inhibits the action of this enzyme (Table 2). The work of du Vigneaud and his group (10) suggests that another route of inactivation of L-penicillamine may be its combination with pyridoxal-5-phosphate to form a thiazolidene carboxylic acid. Finally, another reaction which would be expected to inactivate penicillamine and its analogues is the oxidation to the disulfide. Information is not available as to the extent of the latter reaction.

The many reactions by which cysteine is catabolized in the body are well known (14). The ineffectiveness of cysteine in the treatment of mercuric chloride intoxication and in the excretion of copper in patients with Wilson's disease is probably the result of this rapid catabolism. The effectiveness of penicillamine appears to be due in part to its relative stability to metabolic degradation. This is substantiated by the present experiments with the amino acid oxidases and cysteine desulfhydrase and also by the observation (3) that N-acetyl-DL-penicillamine is superior to DL-penicillamine in protecting rats against the lethal effects of mercuric chloride. As would be expected, the N-acetyl derivative is not active as a substrate of either amino acid oxidase (5). In order to increase the excretion of copper it appears that the α-amino group is necessary, since N-acetyl-DL-penicillamine does not increase copper excretion in patients with Wilson's disease. It is of interest to note that it is also devoid of anti-vitamin B_6 activity (10).

The inference should not be made that any metabolically stable amino acid will protect against metal intoxication. α-Amino isobutyric acid, an amino acid which is not subject to detectable permanent alteration in the body (15), does not protect against mercuric chloride (2). A sulfhydryl group is also necessary since penicillamine disulfide does not increase copper excretion (18).

From the present experiments one can begin to arrive at certain tentative conclusions that may be of aid in designing more effective drugs. As substitution on the

TABLE 2. *Activity of Penicillamine as a Substrate of L-Cysteine Desulfhydrase (5)*

Substrate. 5.0 μmoles of each compound were used	H_2S Produced, μmoles	Activity, %
L-cysteine	3.01	100
L-penicillamine	0.00	0
D-penicillamine	0.00	0
L-cysteine + L-penicillamine	0.53	18
L-cysteine + D-penicillamine	2.07	69

TABLE 3. *Stimulation of Copper Excretion by β-Methyl-β-Ethyl Cysteine*

Date	Medication	24-Hour Urine Excr., ml	Micrograms Copper/ml	24-Hour Copper Excr., mg
5–14	None	1780	0.010	0.018
7–9	0.25 gm β-Me-β-Et-CySH	1460	0.173	0.253
7–10	0.50 gm β-Me-β-Et-CySH	970	0.346	0.336
7–23	1.00 gm β-Me-β-Et-CySH	1280	0.330	0.422
7–24	1.00 gm Penicillamine	1310	0.293	0.384

Abbreviations: Me = Methyl; Et = Ethyl; CySH = Cysteine. On all days before and after the experiment, as well as between 7–10 and 7–23, this female patient with Wilson's disease was receiving daily doses of penicillamine.

β-carbon atom of cysteine increases, the degradation of the molecule by L- and D-amino acid oxidases decreases and its effectiveness in the treatment of heavy metal intoxication, as we have documented elsewhere (2), increases. It is well known (14) that neither the D- nor the L-amino acid oxidase degrades amino acids unless there is a hydrogen attached to the α-carbon atom. The alteration of the cysteine molecule by increased substitution of the β-carbon atom to give β-methyl-β-ethyl cysteine, β,β-diethyl cysteine and other such modifications, in addition to the replacement of the α-hydrogen atom by a methyl, ethyl or larger moiety, might yield drugs of considerably greater activity for the treatment of hepatolenticular degeneration, mercury, and other heavy metal intoxications.

If this hypothesis is correct, β-methyl-β-ethyl cysteine should be as active or more active than penicillamine in stimulating the excretion of copper. Dr. I. Herbert Scheinberg recently tested β-methyl-β-ethyl cysteine in a patient with Wilson's disease. In Table 3 are summarized the results of Dr. Scheinberg's first experiments. This compound, as predicted, increases the urinary excretion of copper. The β-methyl-β-ethyl cysteine we sent to Dr. Scheinberg was a mixture of the four optical isomers. It is conceivable on the basis of our experience with the different activities of the optical isomers of penicillamine in protecting against mercuric chloride intoxication that only one of the four isomers of β-methyl-β-ethyl cysteine may be active in stimulating copper excretion.

A careful study of other metal-binding agents using the various techniques of biochemistry and pharmacology should enable therapists to have the most active and least toxic metal-binding agents available for the treatment of a growing number of clinical disorders associated with increased concentration of metals in the body.

REFERENCES

1. Aposhian, H. V. *Science* 128: 93, 1958.
2. Aposhian, H. V. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 290.
3. Aposhian, H. V. and M. M. Aposhian. *J. Pharmacol. Exp. Therap.* 126: 131, 1959.
4. Aposhian, H. V., R. M. Blair, M. Morris and C. H. Smithson. *Biochim. Biophys. Acta* 36: 93, 1959.
5. Aposhian, H. V. and L. S. Bradham. *Biochem. Pharmacol.* 3: 38, 1959.
6. Aposhian, H. V., M. Morris and M. M. Aposhian. *Abstracts of Papers: 132nd Meeting Am. Chem. Soc.* New York, 1957, p. 93c.
7. Aposhian, H. V. and J. A. Setliff. *Fed. Proc.* 15: 212, 1956.
8. Aposhian, H. V., S. M. Wolff and W. G. Rhea, Jr. *Arch. Biochem. Biophys.* 71: 442, 1957.
9. Boulding, J. E. and R. A. Baker. *Lancet* 2: 985, 1957.
10. du Vigneaud, V., E. J. Kuchinskas and A. Horvath. *Arch. Biochem. Biophys.* 69: 130, 1957.
11. Kuchinskas, E. J. and V. du Vigneaud. *Arch. Biochem. Biophys.* 66: 1, 1957.
12. Kuchinskas, E. J., A. Horvath and V. du Vigneaud. *Arch. Biochem. Biophys.* 68: 69, 1957.
13. Laver, W. G., A. Neuberger and S. Udenfriend. *Biochem. J.* 70: 4, 1958.
14. Meister, A. *Biochemistry of the Amino Acids.* New York: Acad. Press, 1957.
15. Noall, M. W., T. R. Riggs, L. M. Walker and H. N. Christensen. *Science* 126: 1002, 1957.
16. Scheinberg, I. H. and I. Sternlieb. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 275.
17. Walshe, J. M. *Am. J. Med.* 21: 487, 1956.
18. Walshe, J. M. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 265.
19. Wilson, J. E. and V. du Vigneaud. *J. Biol. Chem.* 184: 63, 1950.

GENERAL DISCUSSION

Dr. Foreman: I think that Dr. Aposhian's work answers that very cogent question which has been asked repeatedly during the meeting. Can a chelating agent have a metabolic effect without the involvement of a metal? Here we have penicillamine acting as an anti-vitamin B_6 agent and being a very, very potent, metabolic agent.

Dr. Klaus Schwarz: I have two brief inquiries for Dr. Scheinberg. In any consideration of toxicity versus dietary requirement, the most important question is that of the "therapeutic" spread, or the "therapeutic" index, if one may say so, i.e., the ratio between the daily requirement and the minimum chronic toxic dose of an element. This ratio, of course, is dependent on the form of the compound in which the element is fed. What is really the "therapeutic" spread for copper? What is the daily requirement of the rat or the human for copper, and what is the minimum chronic toxic dose, i.e., the amount which when supplied daily produces toxic symptoms?

The second question is whether copper in the bile is lost for intermediary metabolism or whether there exists an enterohepatic cycle for the element, just as we have for many other constituents of the bile. It is my impression that bile acids, etc., would tend to bind the copper irretrievably, but this is strictly hypothetical, of course.

Dr. Scheinberg: Nobody has done studies, so far as I know, to relate the minimal toxic dose to the daily requirement. It is hard to produce copper toxicity in any animal. I think this is another facet of the fact that we have evolved a built-in mechanism to prevent copper from becoming toxic and this operates effectively with ranges of copper intakes that are very large indeed. In the human being, of course, nobody really knows what the minimal daily requirement is because nobody has ever seen copper deficiency.

There is no evidence for an entero-hepatic circulation of copper, but it probably occurs. If you give Cu^{64} intravenously, you find some in the bile.

Dr. Schubert: Dr. Scheinberg, there appears to be some differences of opinion in the literature as to whether copper levels in the blood do occur in a consistent way following infection or other diseased states. I would like to have your comment on this point.

Dr. Scheinberg: It is true, as Dr. Schubert says, that the serum copper concentration rises in acute infections. It rises in

chronic infections, in pregnancy, in leukemia, in lymphoma, in Hodgkin's disease, in rheumatoid arthritis, and, in fact, in just about every affliction to which man is heir. One must except, however, schizophrenia and other mental illnesses, despite much hullaballoo to the contrary. A few diseases including—kwashiorkor; nephrosis, where there is direct evidence that ceruloplasmin and, therefore, copper are lost in the urine; a disease of infants wherein hypoproteinemia, hypoferremia, and hypocupremia are accompanied by deficiency of ceruloplasmin; and certain conditions, such as, sprue and malabsorption associated with scleroderma, in which the amount of copper absorbed is reduced—are associated with relatively transient deficiency of ceruloplasmin and serum copper compared with the hereditary deficiency in Wilson's disease (Scheinberg, I. H. and I. Sternlieb. *Pharmacol. Rev.* 12: 355, 1960).

Dr. Schubert: Would you comment on the ceruloplasmin levels under stress and the relative roles of cupric and cuprous copper metabolically?

Dr. Scheinberg: Changes in ceruloplasmin have been studied in stress of the types that I have mentioned and with the results that I tried to summarize very quickly.

As for the role of cuprous or cupric copper, I would not like to talk about this without a great deal of thought, and I'm afraid that, even with thought, I still would not know the answer. I do doubt that the oxidase activity of ceruloplasmin has anything to do with its physiological activity. Children who lack ceruloplasmin, such as one little boy I have seen whose liver was normal, have no other detectable abnormality. I wanted to bring this in as some evidence for the fact that ceruloplasmin doesn't have any observable function in the body except, perhaps, to regulate copper metabolism.

Dr. Schubert: These children without ceruloplasmin, how do they respond to stress? Do they show symptoms?

Dr. Scheinberg: The children who have no ceruloplasmin seem happy. This little boy with the normal liver is a delightful, bright, energetic boy whom, I hope, we can prevent from developing Wilson's disease by a regimen of copper depletion.

Let me amend one thing I said. If we bind an atom, such as copper with an atomic weight of 63, into a protein of molecular weight 150,000, the small atom will tend to stay in the vascular compartment, instead of diffusing out of it and into the eyes, the liver, the kidney and other tissues. The function of ceruloplasmin may be that simple: to keep copper from diffusing freely into tissues and, thus, to allow eventual excretion of its copper into the gut. So the form of copper in ceruloplasmin, and its oxidase activity, may be only incidental to the physiologically important fact that the copper is bound to a large molecule which makes it less free to pass through membranes.

Dr. Theodore Hall (Memorial Center, New York): Dr. Scheinberg, you have mentioned individuals who have high tissue copper levels but no clinical symptoms. I suppose some of them have gone on to develop clinical Wilson's disease later. Does the change from asymptomatic to symptomatic correlate with an increase in their already high copper concentrations?

Dr. Scheinberg: The answer to the first part of the question is, "yes." There have been several people described in the literature who had deficiency of ceruloplasmin when first seen (Scheinberg, I. H. and I. Sternlieb. *Pharmacol. Rev.* 12: 355, 1960). They were perfectly normal when the aceruloplasminemia was detected. Most of them already have developed the pathological and clinical findings that define Wilson's disease. But I don't know what their copper levels are, or whether they have increased or decreased with increasing age.

The second part of the question is difficult. We know at least one individual, in whom Wilson's disease is almost certain to develop, who possessed a very high concentration of copper in his liver at 3½ years of age. This is the little boy whose liver appeared essentially normal by biopsy. Yet, this concentration of hepatic copper was three times higher than that of a patient who died of Wilson's disease. This combination of high hepatic copper with a still normal liver suggests, perhaps, that in these patients hepatic copper is high at birth. Maybe it rises *in utero*. Perhaps copper is present in all fetal livers in concentrations greater than in adults, falling after birth in those of us who are not destined to become patients with Wilson's disease.

Dr. Williams: What is the brown color in the Kaiser-Fleischer ring in the eye? Is it cupric cysteine?

Dr. Scheinberg: I don't know.

Dr. Seven: Dr. Scheinberg, several of the things you have said are controversial. Without plunging into a controversy, I would like to state for the benefit of others that there are other groups investigating Wilson's disease with other concepts. I don't have any strong feelings, myself, but would like to mention several points. The changes in color that you observe in the Kaiser-Fleischer ring on treatment, as pointed out by others, are not necessarily related to the removal of copper, and may not be a good index of the efficiency of the copper-binding agent.

Secondly, I think you have not been strong yourself in insisting that the treatment of choice, now, for Wilson's disease is penicillamine. You know that we recently have reviewed a number of cases to which you have contributed yours. The natural history of the disease may be a very strong up and down course. As we discussed in Philadelphia a year ago there are quite a number of patients who do improve and the apparent improvement often is attributed to medication. At this moment, I am not certain that penicillamine is the treatment of choice. I am not sure that any copper-binding agent is the treatment of choice; and I am very much bothered by the natural variations in the course of the disease. There is one group which shows dramatic deterioration from the onset of the symptoms and another group which goes on for years and years—15 to 20 years. However, the metabolic studies in this latter group are the same or even worse by these copper criteria than those who deteriorate rapidly. I don't believe we have the answer to all of these things.

Dr. Scheinberg: It is true that the story of copper metabolism which I presented is partly hypothetical. And I think that there is controversy about some of the points that I mentioned including those that Dr. Seven pin-pointed. But I don't think it is a terribly widespread controversy.

The point about the Kaiser-Fleischer ring, I think, is made chiefly by Dr. Uzman in Boston who, by the way, has contributed to the proof that the Kaiser-Fleischer ring consists of copper (Uzman, L. L. and M. A. Jakus. *Neurology* 7: 341, 1957). He thinks that the appearance of the ring can change without signifying any removal of copper from the ring. Yet, I have seen such a ring virtually disappear by examination with a slit lamp, in about two years of treatment. I can't really believe when this happens that one can seriously doubt that the copper, which everybody agrees causes the ring, has disappeared.

Now, as far as the treatment of the disease goes, it is certainly true, as Dr. Seven said, that the disease has ups and downs. Probably, some examples of dramatic improvement could be

duplicated in some instances in patients without therapy. We have an example of a remarkable spontaneous remission. Before one 13 year old girl was diagnosed as suffering from Wilson's disease, she was found clutching a hydrant in the street unable to walk and unable to talk. She was carried home and admitted a month later to the Neurological Institute of Presbyterian Hospital in New York. By that time, she had recovered speech and the ability to walk and she had had no therapy whatsoever. Yet, it is also true that this disease generally is considered progressive and fatal and spontaneous exacerbations and remissions are not unknown in such illnesses. We have not treated this disease long enough with anti-copper regimens to be certain that we are going to prevent patients from dying or from having their disease progress. But the evidence that I have tried to marshal here is such that if I had Wilson's disease I would certainly take penicillamine in large amounts.

DR. APOSHIAN: I wish to make one brief comment as to the introduction of penicillamine as an oral agent in the treatment of Wilson's disease since it demonstrates another example of a rational approach to therapeutics. John Walshe, who is now a member of the Department of Experimental Medicine at Cambridge University, demonstrated that patients with liver injury excrete penicillamine in the urine after they have received parenteral penicillin. Since penicillamine, a sulfhydryl amino acid, is excreted to some extent in the free sulfhydryl form, it occurred to him that the sulfhydryl moiety in this molecule probably is not metabolized and that, if given orally to patients with Wilson's disease, it might be effective in binding copper and increasing the urinary excretion of this metal. When penicillamine was tested clinically it was effective in this regard (*Am. J. Med.* 21: 487, 1956).

DR. PETERS: I would like to comment relative to the possible toxicity of copper. Since 1954, Dr. Frank Kozelka and I have been doing trace mineral analyses of urine in a host of different neurological and psychiatric conditions with the number of determinations running up into the thousands. We have noted marked increases in copper and zinc urinary excretion in such conditions as delirium tremens, Wernicke's encephalopathy, many of the neuropathies, viral encephalitis, infectious mononucleosis, and periarteritis nodosa, to name a few.

The concentration of copper and zinc in the urine seemed to parallel the severity of the neurological and psychiatric symptomatology with a tendency to return to normal as the patient improved. Some of our highest values have been in cases of acute porphyria during periods of exacerbation. Chronic porphyria patients with cutaneous symptomatology have tended to show predominantly an increase in copper excretion. One of the chronic porphyrics developed during pregnancy what looked like nephritis from which she recovered following delivery of a normal infant. I think this is of interest in view of the toxicity studies done in animals. Following oral chelation with calcium disodium EDTA over a year's time, there has been marked improvement in her photosensitive skin lesions. Her urinary status has returned toward normal.

Another group of patients who tended to show an increase in urinary copper excretion were those epileptic patients who began to develop neurological and psychiatric symptoms following exposure to diphenylhydantoin or Dilantin for periods of time ranging from months to many years. On changing these people to other anticonvulsant drugs, the urinary excretion of copper returned toward normal as the patient improved.

We have made the observation that, when a person is intoxicated with other heavy metals, such as lead, arsenic or mercury, they demonstrate a tremendous increase in the urinary excretion of copper and zinc, in addition to putting out an increased amount of the toxic cations. It is our feeling that the increased excretion of urinary copper and zinc may be as good a guide to the period when a patient is developing toxic symptoms from lead exposure as the coproporphyrinuria which has been used as an index in the past.

Finally, I would like to make one comment on the use of penicillamine. We have observed a very severe allergic response to this compound in one patient who developed severe hives and other symptoms that resembled in every way an allergic reaction to penicillin immediately after his first dose of penicillamine. I wonder if these other penicillamine compounds have the same tendency to produce an allergic response in those individuals sensitive to penicillin.

DR. FOREMAN: Is it correct that your patients had an increase in copper and zinc excretion before the administration of the chelating agents?

DR. PETERS: These people were demonstrating an increased concentration of copper and zinc in their urine prior to any administration of chelating agents. This increased concentration of zinc and copper, on a per liter basis, tended to remain present whether the individual had oliguria or was putting out relatively large amounts of urine. We have expressed these values on a per liter basis just as one would express lead excretion on this basis since 24 hour values for lead, zinc or copper excretion may be quite misleading, whereas the concentration of these compounds on a volume basis tends to remain relatively static, even as the urinary volume increases. A patient with lead intoxication and oliguria actually might put out a low amount of lead on a 24 hour basis, whereas on the per liter basis it would be in the toxic range. As the oliguria diminishes, often with clinical improvement, the urinary concentration of lead continues in the pathological range. If, on the other hand, one simply studies the 24 hour excretion, one could take a normal person, induce a diuresis, and push his 24 hour excretion of lead to a level where some people would consider it to be evidence of toxicity. Obviously, it is not.

DR. COTZIAS: I would like to make one point; in the case of many of these trace elements, the main excretory route is the fecal, not the urinary, route. We all persist in examining mainly the urine. It seems to me it behooves us to set up methods by which we can look at the bile. We have done so relative to manganese but I would like to propose that this be a general preoccupation.

DR. WILLIAMS: Dr. Aposhian, the selectivity between the action of penicillamine and N-acetyl-penicillamine on mercury and copper could be based on the lower coordination of mercury than copper. Mercury tends to prefer *two* linear bonds without chelation, here perhaps to the sulfur of either penicillamine or the N-acetyl compound, while copper is at least four coordinated and chelated, here perhaps to sulfur and nitrogen of penicillamine. Combining the nitrogen with acetyl would reduce the affinity of this group for copper.

Use of chelating agents in treatment of metal poisoning (with special emphasis on lead)

HARRY FOREMAN

Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico

ALTHOUGH IT IS DIFFICULT to estimate the extent to which chelating agents are used in treatment of heavy metal poisonings, use of these agents has become the therapy of choice in a number of them—i.e., calcium ethylenediaminetetraacetate (Ca EDTA) in lead poisoning, dimercaprol (BAL) in arsenic poisoning, etc. Most of such cases, probably up to a thousand or so per year (39), are treated with these drugs. So well established has this form of therapy become that, by and large, uncomplicated cases involving the use of these agents for more commonly seen metal poisonings, i.e., lead, are no longer reported in the literature.

Of the metals which produce noxious effects on entry into the body, lead is the most interesting from the viewpoint of chelation therapy, partly because most of the clinical experience gained with such agents has been obtained with lead and partly because the therapy of lead poisoning illustrates very well the principles and problems involved in the *in vivo* use of chelates. Clinical experiences in the use of chelating agents for the treatment of toxicity from metals other than lead have been quite limited, largely owing to the lack of case material, but in part also because of ambiguity as to the effectiveness of these agents. Many compounds have been suggested. Table 1 summarizes the agents which have been tried clinically and presents an evaluation of their use as judged at the present time. Studies in animals are given in a few instances where the results were of special interest and where no experience in human beings has yet been reported. Although the information presented in the table represents a rather thorough survey of the literature, no claim is made for completeness, particularly with respect to citations. Generally, references are made to summary or review papers and to the most recent work available.

Treatment rationale. One of the first questions that arises in a consideration of the use of chelating agents concerns the actual necessity for a definitive therapeutic agent which can effect the removal of the metal from the body. In the case of lead, for example, there is a school of thought which holds that except for supportive therapy in the alleviation of acute symptoms, an individual with lead poisoning is just as well off not treated. This approach to the lead poisoning problem is based upon the reasoning that lead poisoning is a self-limiting disorder, in that removal of the individual from exposure to lead will, in time, result in the disappearance of symptoms and the restoration of well-being. The disappearance of toxicity comes about because the bulk of lead in the body eventually becomes tied up in the skeleton, wherein supposedly it is rendered harmless. The effectiveness of EDTA in accelerating the disappearance of symptoms and signs of toxicity has contributed significantly towards dispelling this more conservative approach to the management of lead cases.

Actually, "dormant" lead in the skeleton is not entirely harmless. It has been reported, particularly in children (15) but also in adults (3), that the symptoms of lead poisoning recur in individuals who have had excessive absorption of lead and then at some time later undergo stress such as febrile illness, severe malnutrition, some chronic diseases, or severe injury. For this reason alone, it would be desirable to rid the body of as much lead as possible, if the means for doing this are available, convenient, and harmless when done properly.

In support of active treatment, a most interesting series of cases has been informally reported by Bell (6), wherein several diagnostic problems with neurological complaints turned out to be lead poisoning. Interestingly enough, the use of EDTA in these cases brought about relief from symptoms and disability which had persisted for as long as 20 years previously. In several of these cases, there had been no lead exposure for many years so that lead poisoning was not considered likely. The diagnosis was made by the so-called provocative EDTA test. In this test, a dose of EDTA [the size depending upon by whom it is done (5, 6, 21, 54, 70, 73)] is given intravenously and 24-hour urine specimens are collected and assayed for lead. Generally speaking, an outpouring of over 1 mg of lead in 24 hours is considered indicative of an excessive body burden of lead. Uncontaminated individuals given this test range far below this amount. In Bell's cases, repeated courses of EDTA over a period of 1½ to 2 years were required before the well-being of the individuals was completely restored. These cases and the course of their treatment emphasize an import-

TABLE 1. *Chelating Agents Used for Treatment of Metal Poisoning*

Metal	Therapeutic Agent	Comments
As	BAL	Most widely accepted chelate therapy. Extensive clinical experience verifies the value of drug in hastening disappearance of exfoliative dermatitis and neurological symptoms (51). Not useful in arsine poisoning.
Be	Aluminon† (58)	Successful clinical application has not been reported.
	Ca EDTA	Brings forth small increase in Be excretion. Clinical usefulness is still not proven (18).
Ca	Na EDTA (65)	While drug is effective in lowering serum Ca in hypercalcemia, effect is transitory and has doubtful therapeutic usefulness (24, 37).
Cd	BAL	Studies suggest use of the drug is contraindicated because of nephrotoxicity produced by Cd complex (33).
	Ca EDTA	Use is controversial (29, 42, 63).
Co	Ca EDTA	Drug hastens excretion of Co (13). Counteracts polycythemic action of Co in animals.
Cr	Ca EDTA	Useful in an ointment to promote healing of chronic ulcers (47).
Cu	BAL (74)	Chelate drugs are reported of value in alleviating neurological symptoms in Wilson's disease but have no effect in altering liver function. Uzman (74) questions any clinical improvement attributed to agents promoting copper diuresis.
	D,L-Penicillamine (57, 76)	
	DMTAC† (74)	
Fe	BAL (25, 60)	Fe excretion is increased in cases of iron toxicity but the clinical course is apparently not altered. Intravenous chelating agents may be of limited value in transfusional hemosiderosis but are not, at present, a substitute for phlebotomy in primary hemochromatosis (27).
	Ca EDTA (17, 62)	
	Versenol† (59)	
Hg	BAL (45)	Effective if given early.
	Ca EDTA	Claims are confusing. One group believes drug of value in chronic Hg poisoning (78); another does not (34).
	N-Acetyl-D,L-penicillamine (2)	Potentially useful drug for oral treatment of mercury intoxication.
Mn	EBAA (30); CHENTA† (67)	Relatively poor in effecting enhanced excretion.
	Ca DTPA† (30); Ca EDTA	Enhances Mn excretion markedly. Aids in alleviation of symptoms (50).
Ni	Ca EDTA	No value (77).
	Dithiocarb†	Has been found effective in promoting well-being in cases of Ni carbonyl poisoning, as well as decreasing mortality in animals from inorganic Ni (66).
Pb	D,L-Penicillamine	Effective by mouth in hastening Pb excretion, but no extensive clinical trial as yet (10, 36).
	BAL	Not of value (36).
	Ca DTPA (31)	Effective in hastening excretion; no extensive clinical trial.
	Ca EDTA	See text.
Th*	Ca DTPA; Ca EDTA (79)	Used in one case of Th exposure. Results were not amenable to evaluation.
Tl	Ca EDTA (1, 72)	Agent hastens excretion of metal but apparently does not alter clinical course.
	BAL (52)	Of no value in altering excretion or clinical course.
U*	Ca EDTA (23); Ca DTPA (19)	Raises LD_{50} markedly in experimental animals.
V	BAL (64)	No effect on excretion.
	Ca EDTA (49)	Enhances excretion in experimental animals.
W	BAL (46); Ca EDTA (63)	Enhances excretion in experimental animals.

* Although Th and U are radioactive, their specific activity is so low that the chemical toxicity is manifest at lower doses than the radiation effect; hence the inclusion of these metals in this Table. † (DMTAC, 2,2-Dimethylthiazolidene-4-carboxylic acid; Versenol, N-Hydroxyethylethylenediaminetriacetic acid; DTPA, Diethylenetriaminepentaacetic acid; Dithiocarb, Diethyldithiocarbamate; EBAA, bis-α,α'-(*o*-hydroxyphenyl)ethylenediaminediacetic acid; CHENTA, 1,2-cyclohexanediamine-N,N,N',N'-tetraacetic acid (CDTA); Aluminon, ammonium aurinetricarboxylate.)

ant aspect of lead poisoning, namely, that the symptoms associated with the disorder do *not* necessarily disappear after the removal of the exposure and can persist for very long periods of time, and that it may be necessary to persist in treatment for long periods of time before a desirable outcome can be effected.

Childhood lead poisoning. The greatest need for improvement in therapeutic techniques for lead poisoning lies in childhood cases, for it is here that the effects of lead poisoning are most distressing. Characteristically, childhood cases of lead poisoning occur in the summer time in youngsters aged 1 to 4, who live in depressed urban areas. The lead is accumulated from ingestion of paint or plaster from surfaces covered with old paint in the process of flaking off. Gram quantities of lead can be consumed in a relatively short time under these conditions. The symptoms associated with such cases range from mere dullness and lethargy, emotional instability, antisocial behavior, and anorexia to profusive vomiting, ataxia, convulsions, stupor, and coma. Characteristically, the child is pallid and on entry into the hospital is found to exhibit anemia, glucosuria, and a well delineated radiopacity in the metaphyseal ends of the long bones, which is often helpful in the diagnosis. Coproporphyrinuria is usually present. Urinary levels range from so-called "normal" levels of below 80 μg per 24 hours up to an order of magnitude greater. The urine excretion levels are not related to the severity of symptoms. The course of the disease is unpredictable and frequently fatal. Not uncommonly, a child in apparently fair condition will suddenly convulse, pass into coma, and die.

The first cases of the treatment of lead poisoning with EDTA were by Bessman *et al.* (8, 9) in a series involving

children who had absorbed various quantities of lead from ingestion of paint in houses in the Washington, D. C., area. Four of the cases showed severe symptoms of encephalopathy with convulsions and coma, and 3 of them presented milder symptoms of vomiting and ataxia. Treatment with EDTA was followed by rapid alleviation of symptoms in 6 of the cases so that improvement was noticeable by 24 hours and the children were apparently well in a few days. The one unfortunate case had entered the hospital with severe convulsions after a history of repeated convulsive episodes over the previous 2 weeks. She was given 0.5 gm of calcium EDTA intravenously. She brightened and in 6 hours was conscious and taking fluids by mouth. Then, a few hours later, she suddenly became convulsive, comatose, and died. Since that time, reports by others (4, 16, 32, 38, 41, 68) have presented similar results in the treatment of acute lead encephalopathy. In a number of these cases, even the most severe symptoms of convulsions and coma were alleviated after 2 days of treatment. In all cases, the treatment was accompanied by an outpouring of lead in the urine, ranging from 20- to 60-fold greater than the pretreatment excretion levels, and, in some cases, as much as 30 mg of lead were removed in a 5-day treatment period.

For an adequate evaluation of these cases, it is necessary to compare them with untreated cases. The courses of a number of untreated cases have been summarized by Ennis and Harrison [Table 2 (26)]. As can be seen, severe lead encephalopathy in children is a dreadful disease with a 65 per cent mortality and even in mild cases, of drastic consequences to the survivors. The residual injury persisting in the majority of the survivors is of serious consequence: blindness, idiocy, and hemiplegia in the worst cases, and ataxia, impaired mental powers, dullness, etc., in the less severely involved individuals. One of the most valuable studies to be reported is that of Chisolm and Harrison (20), wherein the course of events following the treatment of lead encephalopathy cases with EDTA is compared with that in a similar series which had been treated with BAL (Table 3).

It can be seen from an intercomparison of these 3 similar series that while there is a significant reduction in mortality following treatment, the mortality rate is still distressing even following treatment. When death occurred, it usually happened within 24 hours after the start of treatment. The effects of lead on brain tissues are fulminating, and apparently not sufficient time is available in the severely affected cases for an adequate course of therapy. The solution to this problem does not necessarily lie in therapy but in earlier diagnosis, so that therapeutic measures can be instituted before the situation becomes desperate. While the extent to which the reduction of mortality as the result of treatment is disappointing, the apparent diminution in sequelae in treated cases over those untreated indicates the desirability of continuing the use of EDTA in the therapeutic regimen. This conclusion is borne out in a subsequent study by Bradley and Baumgartner (12).

TABLE 2. *Mortality Rate and Incidence of Permanent Neurological Residua in Cases of Lead Encephalopathy Summarized from the Literature [Reference 26]*

Author	Type of Encephalopathy	No. Cases	% Mortality	No. Survivors	No. Residual Injuries
Holt*	Severe	20	13 (65)	7	6
McKhann and Vogt†	All types	45	11 (25)	34	12
Byers and Lord‡	Mild	20	0	20	19
Kanner§	All types	59	20 (34)		
	Severe	29	20 (65)		
Ennis and Harrison	All types	31	12 (36)	19	13
Control Group	Severe	18	12 (66)	6	6

* Holt, L. E. *Am. J. Dis. Child.* 25:229, 1923. † McKhann, C. F. and E. C. Vogt. *J.A.M.A.* 101: 1131, 1933. ‡ Byers, R. K. and E. E. Lord. *Am. J. Dis. Child.* 66:471, 1943. § Kanner, L. In: *Child Psychiatry* (2nd ed.). Springfield, Ill.: Thomas, 1948, p. 290.

TABLE 3. *Acute Lead Encephalopathy—a Comparison of Cases Treated with Calcium EDTA with Those Treated with BAL**

Therapy	No. Cases	Encephalopathy	No. Died	No. Survived	No. Followed 1+ years	CNS Sequelae	
						None	Present
Calcium EDTA	22	Mild	0	22	16	15	1
	14	Severe	5	9	9	7	2
Totals	36		5	31	25	22	3
BAL	18	Mild	0	18	7	5	2
	15	Severe	5	10	3	2	1
Totals	33		5	28	10	7	3

* Adapted from Chisolm and Harrison (20).

Adult lead poisoning. By and large, lead poisoning in adults occurs from occupational exposure. The toxicity produced in adults generally is not nearly so severe as that seen in children and is manifest in several forms. The symptoms can be grouped as those related to the alimentary tract, the neuro-muscular system, and those associated with encephalopathy. In almost all cases, the bone marrow is involved with resultant anemia and coproporphyrinuria. Typically, the lead-intoxicated worker is often dulled and irritable with a tendency to complain about headache and fatigue, and not infrequently about constipation and gastrointestinal cramps. The more severely involved cases will exhibit muscular weakness (palsy), tremors, and ataxia, and if very severe, convulsions and coma. Urine lead levels generally are not related to the severity of the symptoms and may range from below levels associated with uncontaminated individuals to milligram quantities excreted daily. Coproporphyrinuria is almost uniformly present, and it is believed by some to provide a good index to the severity of the toxicity (56).

Starting in 1953 and continuing through to the present, reports in the literature on over 100 cases (5–7, 14, 21, 35, 43, 48, 53, 55, 56, 61, 75) indicated EDTA

produces marked alleviation of symptoms within short periods of time after treatment is started. Many times, colic disappears within 2 hours (75). By way of comparison, in the reports on untreated individuals colic may persist as long as 5 to 6 weeks after removal from lead exposure (3). A feeling of well-being is often restored by 48 hours. Muscular weaknesses and tremors disappear after 4 to 5 days of treatment. The coproporphyrinuria, stipple cells, and lead lines tend to remit within 4 to 9 days, quite often with noticeable increase in hemoglobin and red cell count by 10 days. The treatment is always accompanied by an outpouring of lead in the urine 10- to 60-fold greater than the pretreatment level. Up to 20 mg of lead have been excreted in a single day (61).

Tetraethyl lead. Another aspect of the lead poisoning problem is the toxicity resulting from exposure to tetraethyl lead. In several respects, this is a disease similar to that of lead poisoning in children in that symptoms are largely neurological and often appear in an acute and fulminating manner. The course is variable with unexpected and unexplained exacerbations and remissions, and the mortality rate is high. Fortunately, unlike childhood lead poisoning, recovery when it does occur is complete with apparently little demonstrable residual damage. The administration of both calcium EDTA and BAL has been reported in two series of cases of tetraethyl lead poisoning (11, 40). In each instance, the administration of the drug was accompanied by an increase in lead excretion. Because of the variable course of the disease and the relatively few cases involved, it was not possible to evaluate the drug with respect to the effect on well-being.

Discussion. What has been learned over the past 10 years about *in vivo* chelation for the treatment of metal poisoning? Relatively little, and there are many important questions still remaining to be answered.

The development of effective agents for mobilizing metals from the body has raised many questions about the disease process of metal poisoning itself, many of which have been considered in the past but are now brought into focus with increased interest and need for solution. For instance, it has now become pertinent to know more precisely the size of the body burden of metal in an individual so that it might be possible to establish the proportion of metal in the body that is mobilized by treatment. Even more desirable is information on metal levels that are associated with tissue damage in specific organs. Other information on the behavior of metals in the body, which has now become cogent, is the transfer rates of metal ions from one body compartment to another (i.e., from tissue cells to interstitial fluid to the blood to intraluminal cavities, etc.) and the effect of the introduction of chelating agents into the body on these rates. If one could establish the rate-determining step in the course of events, then attempts could be directed toward the design of a chelating agent with specific capability for hastening transfer across the particular membrane involved. Of interest also are the relative binding affinities of the various tissues for metal ions as compared to the binding affinities with chelating agents under physiological conditions. Some information on this is reported by Teisinger *et al.* (69, 71) on the competition between calcium EDTA and lead bound to the red cells and to liver tissue.

For a more adequate understanding of the use of chelating agents, much remains to be learned about the behavior of the agents themselves within the body. Although a little has been learned about the ebb and flow of calcium EDTA, little is known about the kinetics of the other metal chelates. Moreover, competition of the various endogenous metals for the chelating agent in body fluids is poorly understood. We are still in the dark as to the long-term effects of the administration of chelates in their depletion of the body of trace metals. We have learned a little about the nephrotoxicity of some of the chelating agents, although we know nothing about the mechanism by which these changes are brought about. Reports are now appearing in the literature (22, 80) indicating an inhibition of iodine uptake by the thyroid during the course of administration of chelating agents. It is postulated that the antithyroid action of thiouracil is mediated by chelation of copper in the thyroid (44).

After all this time, there is still considerable ambiguity as to the optimum dose of chelating agents, size, frequency, rate of injection, and mode of administration; even of the most widely used drug, calcium EDTA. The primary limitation on the size of dose that can be given is the nephrotoxicity of the drug. Foreman *et al.* (28) have shown that, if given repeatedly, calcium EDTA will produce kidney lesions and that the larger the dose the sooner the lesion develops. Because of this finding, an upper dose of 50 mg per kg per day for 5 days with a minimum of 2 days' rest was recommended. This would appear to be the optimum dose. However, a number of workers have shown that there is no simple relationship between size of dose and effectiveness, and that smaller doses have a greater relative efficiency. Bell (6) found that 3 gm doses brought forth only 3.3 times as much of an increase in lead excretion as did a 0.1 gm dose. Leckie *et al.* (43) found that a 4 gm dose was no more effective than a 2 gm dose and that a 4 gm dose brought forth only 2.5 times as much lead as did a 50 mg dose. Moreover, the rate at which a dose is given varied the effectiveness of the drug. Giving the dose over a 6-hour period achieved double the urinary lead excretion of the same dose given in 1 hour. Because of the rapid turnover time of the drug (1½ hours) in the body, one would expect that divided doses over the day would give a more constant blood level than the same dose given in a single injection. Actually, a single dose per day regimen has been found to be as satisfactory as any other scheme. This is all puzzling and may be cleared up when more is known about the behavior of the lead chelate in the body. A possible explanation of the relatively greater efficiency of lower doses is that somewhere in the trans-

port chain there is a membrane whose transfer potential for calcium EDTA is saturated at low concentrations.

It is of interest to consider how presently used chelates might be improved to perform their desired function more adequately. Simple changes in the form of the drug available would be helpful, particularly those which would allow flexibility in the route of administration. Intravenous administration is the route now most commonly employed. Most of the time this is an inconvenient way to give a drug. Oral administration would be the most desirable method, but this route of administration has not been found to be very effective. Subcutaneous injections have been used successfully in infants by Chisolm (20) and Kneller (41), but this is not generally applicable to adults. The best method for many reasons is intramuscular administration. It is not used widely at this time because the drug is painful given in this fashion. However, mixed with a local anesthetic, it can be given without discomfort. Of interest might be an aerosol, which would allow administration of the drug by inhalation.

In view of the apparent inability of chelates to elute metals fixed in tissue as compared to the ease with which metals can be removed from the blood, it might be fruitful to design chelates capable of entering the cells, particularly in lipid-rich tissue such as nerve tissue and the bone marrow. Such drugs might effect the rapid action apparently necessary in lead encephalopathy in children.

Summary. Chelating agents have a fairly well established place in the treatment of lead, arsenic, chromium, manganese, thorium, uranium, and nickel poisoning. The value of their hastening the excretion of copper in Wilson's disease is in dispute. In beryllium, iron, calcium, and thallium toxicity, it is not likely that these agents are useful at present. Their application is considerably contraindicated in cadmium exposure.

REFERENCES

1. Abahory, C., R. Truhaut and L. Boudere. *Ann. med. leg.* 38: 245, 1958.
2. Aposhian, H. V. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 290.
3. Aub, J. C., L. T. Fairhall, A. S. Minot and P. Reznikoff. *Lead Poisoning.* Baltimore: Williams and Wilkins, 1926, p. 7.
4. Belknap, E. L. *Indust. Med.* 21: 305, 1952.
5. Belknap, E. L. and M. C. Perry. *A.M.A. Arch. Indust. Hyg. Occup. Med.* 10: 530, 1954.
6. Bell, R. F. *Indust. Med.* 28: 153, 1959.
7. Bell, R. F., J. C. Gilliland, J. R. Boland and B. R. Sullivan. *A.M.A. Arch. Indust. Health* 13: 366, 1956.
8. Bessman, S. P., H. Ried and M. Rubin. *M. Ann. District of Columbia* 21: 312, 1952.
9. Bessman, S. P., M. Rubin and S. Leikin. *Pediatrics* 14: 201, 1954.
10. Boulding, J. E. and R. A. Baker. *Lancet* 2: 985, 1957.
11. Boyd, P. R. *Lancet* 1: 181, 1957.
12. Bradley, J. E. and R. J. Baumgartner. *J. Ped.* 53: 311, 1958.
13. Brendel, R., V. Swayne, R. Preston, J. M. Beiler and G. J. Martin. *J. Am. Pharm. A. (Scient. Ed.)* 42: 123, 1953.
14. Browne, R. C. *Lancet* 1: 775, 1955.
15. Byers, R. K. and E. E. Lord. *Am. J. Dis. Child.* 66: 471, 1943.
16. Byers, R. K. and C. Maloof. *A.M.A. Am. J. Dis. Child.* 87: 559, 1954.
17. Cann, H. M. and H. L. Verhulst. *A.M.A. J. Dis. Child.* 99: 688, 1960.
18. Cash, R., R. I. Shapiro, S. H. Levy and S. M. Hopkins. *New England J. Med.* 260: 683, 1959.
19. Catsch, A. *Klin. Wchnschr.* 37: 657, 1959.
20. Chisolm, J. J., Jr. and H. E. Harrison. *Pediatrics* 19: 2, 1957.
21. Cotter, L. H. *J.A.M.A.* 155: 906, 1954.
22. Current, J. V., I. B. Hales and B. M. Dobyns. *J. Clin. Endocrinol.* 20: 13, 1960.
23. Dagirmanjian, R., E. A. Maynard and H. C. Hodge *J. Pharmacol. & Exper. Therap.* 117: 20, 1956.
24. Dudley, H. R., A. C. Ritchie, A. Shilling and W. H. Baker. *New England J. Med.* 252: 331, 1955.
25. Edge, N. D. and G. F. Somers. *Quart. J. Pharm. Pharmacol.* 21: 364, 1948.
26. Ennis, J. M. and H. E. Harrison. *Pediatrics* 5: 853, 1950.
27. Figueroa, W. G. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 146.
28. Foreman, H., C. Finnegan and C. C. Lushbaugh. *J.A.M.A.* 160: 1042, 1956.
29. Friberg, L. *A.M.A. Arch. Indust. Health* 13: 18, 1956.
30. Fried, J. F., A. Lindenbaum and J. Schubert. *Proc. Soc. Exper. Biol. & Med.* 100: 570, 1959.
31. Fried, J. F., J. Schubert and A. Lindenbaum. *A.M.A. Arch. Indust. Med.* 20: 473, 1959.
32. Giles, H. McC., C. J. Moore and B. M. Still. *Lancet* 1: 183, 1955.
33. Gilman, A., F. S. Philips, R. P. Allen and E. S. Koelle. *J. Pharmacol. & Exper. Therap.* 87 (Suppl.): 85, 1946.
34. Glomme, J. and K. H. Gustavson. *Acta med. scandinav.* 164: 175, 1959.
35. Hardy, H. L., H. B. Elkins, B. P. W. Ruotolo, J. Quinby and W. H. Baker. *J.A.M.A.* 154: 1171, 1954.
36. Harris, C. E. C. *Canad. M. A. J.* 79: 664, 1958.
37. Holland, J. F., E. Danielson and A. Sahagian-Edwards. *Proc. Soc. Exper. Biol. & Med.* 84: 359, 1953.
38. Karpinski, F. E., Jr., F. Rieders and L. S. Girsh. *J. Pediatrics* 42: 687, 1953.
39. Kehoe, R. A. *J.A.M.A.* 157: 341, 1955.
40. Kitzmiller, K. V., J. Cholak and R. A. Kehoe. *Arch. Indust. Hyg.* 10: 312, 1954.
41. Kneller, L. A., H. S. M. Uhl and J. Brem. *New England J. Med.* 252: 338, 1955.
42. Kocher, Z., V. Eybl and J. Sykora. *Arch. internat. pharmacodyn.* 115: 164, 1958.
43. Leckie, W. J. H. and S. L. Tompsett. *Quart. J. Med.* 27: 65, 1958.
44. Liberman, D. *Nature, London* 164: 142, 1949.
45. Longcope, W. T. and J. A. Luetscher, Jr. *J. Clin. Invest.* 25: 557, 1946.
46. Lusky, L. M., H. A. Braun and E. P. Laug. *J. Indust. Hyg. Toxicol.* 31: 301, 1949.
47. Maloof, C. C. *A.M.A. Arch. Indust. Health* 11: 123, 1955.
48. Markus, A. C. and A. G. Spencer. *Brit. M. J.* 2: 883, 1955.
49. Mitchell, W. G. *Proc. Soc. Exper. Biol. & Med.* 83: 346, 1953.
50. Penalver, R. *A.M.A. Arch. Indust. Health* 16: 64, 1957.
51. Peters, R. A. *Brit. M. J.* 2: 520, 1947.
52. Richeson, E. M. *Indust. Med.* 27: 607, 1958.
53. Rieders, F. *Fed. Proc.* 14: 382, 1955.
54. Rieders, F. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 143.
55. Rieders, F., W. G. Dunnington and H. Breiger. *Indust. Med.* 24: 195, 1955.
56. Ruotolo, B. P. W. and H. B. Elkins. *A.M.A. Arch. Indust. Hyg. Occup. Med.* 9: 205, 1954.
57. Scheinberg, I. H. and I. Sternlieb. In: *Metal-Binding in*

Medicine, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 275.
58. Schubert, J., M. R. White and A. Lindenbaum. *J. Biol. Chem.* 196: 279, 1952.
59. Seven, M. J., H. Gottlieb, H. L. Israel, J. G. Reinhold and M. Rubin. *Am. J. M. Sc.* 228: 646, 1954.
60. Shoss, J. *J. Pediatrics* 44: 77, 1954.
61. Sidbury, J. B., Jr., J. C. Bynum and L. L. Fetz. *Proc. Soc. Exper. Biol. & Med.* 82: 226, 1953.
62. Sisson, T. R. C. and W. R. Bronson. *A.M.A. J. Dis. Child.* 96: 463, 1958.
63. Sivjako, K. I. and H. A. Braun. *Tox. Appl. Pharmacol.* 1: 602, 1959.
64. Sjoberg, S. *A.M.A. Arch. Indust. Hyg.* 3: 631, 1951.
65. Spencer, H., V. Vankinscott, I. Lewin and D. Laszlo. *J. Clin. Invest.* 31: 1023, 1952.
66. Sunderman, F. W. and F. W. Sunderman, Jr. *Am. J. M. Sc.* 236: 26, 1958.
67. Sykora, J., Z. Köcher and V. Eybl. *A.M.A. Arch. Indust. Health* 21: 24, 1960.
68. Tanis, A. L. *A.M.A. J. Dis. Child.* 89: 325, 1955.
69. Teisinger, J., K. Lustenec and J. Srbova. *A.M.A. Arch. Indust. Health* 17: 302, 1958.
70. Teisinger, J. and J. Srbova. *Brit. J. Indust. Med.* 16: 148, 1959.
71. Teisinger, J., R. Zumova and I. Zizula. *A.M.A. Arch. Indust. Health* 17: 295, 1958.
72. Thiers, H., A. Badinand, J. Condert, A. Boucherle, D. Colomb and J. Fayolle. *Ann. med. leg.* 38: 261, 1958.
73. Unseld, D. W. *Klin. Wchnschr.* 36: 328, 1958.
74. Uzman, L. L. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 269.
75. Wade, J. F., Jr. and J. F. Burnum. *Ann. Int. Med.* 42: 251, 1955.
76. Walshe, J. M. *Am. J. Med.* 21: 487, 1956.
77. West, B. and F. W. Sunderman. *A.M.A. Arch. Indust. Health* 18: 480, 1958.
78. Woodcock, S. M. *Brit. J. Indust. Med.* 15: 207, 1958.
79. Young, W. N. and H. A. Tebrock. *Indust. Med.* 27: 229, 1958.
80. Zizine, L. *Compt. rend. soc. biol.* 152: 31, 1958.

DISCUSSION

Value of calcium disodium ethylenediaminetetraacetate and British anti-lewisite in therapy of lead poisoning

ROBERT A. KEHOE
University of Cincinnati College of Medicine, Cincinnati, Ohio

British anti-lewisite (BAL) may be discarded summarily, in this relationship, at the outset, without the necessity of presenting here the physiological evidence that it is essentially without value, or discussing the practical disadvantages and the dangers, perhaps, of its use for this purpose (4). Concerning calcium disodium ethylenediaminetetraacetate (hereafter referred to as $CaNa_2EDTA$), opinions expressed in this discussion will run counter to the current of enthusiasm for its use in the therapy of lead poisoning. A favorable view is expressed, but one that does not admit that this is "the specific remedy" or that it is adequately efficacious. Indeed, it seems to me to be likely that this compound will be superseded by a more effective chelating agent or that resort to some other principle may better accomplish the therapeutic conquest of this disease.

$CaNa_2EDTA$ is useful in the treatment of lead poisoning; but it does not act with sufficient promptness or certainty in the relief of the symptoms of lead intoxication, and it does not save life when the threat of death is imminent. Rather, strange as it may seem, it is hailed by those whose approval would be expected to be derived from its clinical efficacy, because of a physiological effect of a somewhat theoretically beneficial type. The administration of $CaNa_2EDTA$ hastens the elimination of lead from most of the soft tissues of the body, and, thereby accelerates, indirectly, its loss from the skeleton as well. Of this, there is no doubt. It follows, therefore—or does it?—that it lessens the toxic effects or shortens their duration. Let us consider some of the facts on which our judgment must be based.

Great caution is required in appraising the efficacy of the medical therapy of a disease that is rarely fatal and is highly variable in the severity of its manifestations and in the duration of disability. The predominant form of lead poisoning in our time, among adults, is that of a disturbance of the alimentary tract of variable severity, accompanied often, but not always, by certain hematological abnormalities. This disease, following the termination of excessive exposure to and absorption of lead.

usually subsides spontaneously. Certain acute symptoms —obstipation and colic, chiefly—may require symptomatic therapy for their relief, but aside from these manifestations of intoxication, in association with inanition and weakness and, occasionally, a somewhat persistent and partially disabling anemia, the illness is self-limited, and with the normal metabolic elimination of excessive quantities of lead from the body, the episode of intoxication is terminated, usually without sequelae. Under certain comparatively infrequent circumstances, the outstanding feature of the disease is a neuromuscular dysfunction characterized usually in the adult by excessive weakness or paralysis of extensor muscles in the upper extremity. This form of the disease is likely to be much more prolonged, but is unpredictably so. In an occasional adult, under very severe conditions of exposure, and in an astonishing proportion of cases among small children (up to or somewhat beyond the age of three), the primary presenting manifestation of lead intoxication is a convulsion or an acute mental disturbance. When the brain is severely involved, these manifestations persist, mount in severity, and often terminate in death.

In view of the characteristics of the most common form of the disease in the adult, especially in association with occupational exposure to lead, it is difficult, to say the least, to assess the merits of medical therapy. It would seem to be logical, therefore, to base one's critical judgment of the efficacy of a specific therapeutic agent or regimen, on its ability to cure the more severe forms of the disease and to reduce materially the numbers of fatal cases. There may be other benefits to be achieved by therapeutic efforts, and in this instance there is good reason to believe that this is the case, but the fact remains that unless the tragic consequences of the more severe forms of lead intoxication can be brought under control, medical therapy is sadly inadequate. The facts in this respect are all too evident. The child suffering from acute encephalopathy due to the absorption of lead, and threatened with death, may be benefited to some extent by the administration of $CaNa_2EDTA$, although in some instances there appears to be an immediate worsening of the condition (5), but surgical intervention appears to be required as a life-saving measure (5). It may be argued that this is true only because medical therapy has been undertaken too late in the disease when the intracranial derangement has reached a point which prevents access, mechanically, on the part of the chelating agent, *via* the blood stream, to the affected brain or when the damage to the brain has come to be irreversible. All explanations aside, the fact of the failure is sufficient in itself.

There is some evidence, on the other hand, that when $CaNa_2EDTA$ has been used judiciously and the acutely poisoned child survives (whether because of or independent of its use), the likelihood of serious sequelae is less, and the duration of illness is shortened (1). There is sufficient reason, therefore, for employing this therapy, along with other available means, until and unless some more effective therapeutic agent has been found.

It has been intimated previously that the use of $CaNa_2EDTA$ may be beneficial in lead poisoning apart from the question of the immediacy or reliability of its relief of acute toxic effects, and reference has been made to its ability to hasten the elimination of lead from the body. Despite our suggestion that the latter physiological effort is only "theoretically beneficial," it is not to be regarded lightly. Whatever may be the mechanism or site whereby or wherein lead induces a functional disturbance, there is an effective concentration of lead in the body as a whole without which no such disturbance occurs. The inactivation of lead by chelation does not seem to occur promptly (or perhaps not at the right site) since the functional disturbance tends to persist, but the eventual elimination of lead permits normal function to be resumed (when there is no irreversible damage), and the hastening of elimination should, therefore, be beneficial in time, at least. It appears that illness and disability are terminated earlier by the proper administration of this chelating agent.

There is practical benefit in the use of $CaNa_2EDTA$ in occupational lead poisoning, however, even if the course of the intoxication is but little affected, for one of the most troublesome features of the disease, from the occupational standpoint, is the fact that abnormal quantities of lead remain in the body for considerable periods of time after the termination of occupational exposure to lead, and after the signs and symptoms of intoxication have disappeared. An excessive body burden of lead is a source of hazard to the man who returns to work involving potentially dangerous exposure to lead, since because of it, under the same conditions, he will reach a dangerous level of absorption in a shorter period of time than was required initially.

When should such a man return to work? (It is all very well to say that he should not resume dangerous work, but many of the lead-using industries continue to be unnecessarily dangerous, and the hard facts of economic life, plus the special skills or the occupational habits of workmen, lead to their resumption of demonstrably dangerous jobs.) There is need to emphasize the duty of the physician in private practice to protect his patient, so far as possible, against this risk, by not permitting him to return into the occupational environment which was responsible for his illness, until some degree of freedom from danger has been provided. If this cannot be achieved by seeking the correction of hazardous conditions (in which he may be wholly unsuccessful), the least that can be done is to assure himself that the lead in his patient's body has been reduced to a level which furnishes some margin of safety for a time. This is a necessary part of the therapy of occupational lead poisoning, for the patient is not ready to return to his former work until this consequence of his occupational exposure to lead has been dealt with. It can be dealt with successfully by checking the extent of the elimination of lead from the body from time to time until the concentration

of lead in the blood has declined to the upper normal level. Since the laws regulating the compensation of workman for occupational disease take no cognizance of this situation, having been designed in relation to symptomatic illness and overt disability rather than to any form of potential disability that requires medical handling for its avoidance, there may well be a proper place here for a well chosen and carefully controlled regimen of therapy which will hasten the normal processes of elimination.

There is a similar and more imperative rationale for the proper medical handling of lead poisoning in childhood. It is not enough to treat such cases of poisoning, and then to permit the child to return into the same environmental situation that was productive of his illness. Whatever may be done otherwise, the least that the physician may be expected to do is to aid in the avoidance of a prompt repetition of the illness by securing an adequate reduction in the quantity of lead that remains in the body of a child whose acute symptoms of lead intoxication have subsided. It is important in this connection to be aware of the fact that such symptoms may subside, despite the persistence of a dangerous quantity of lead in the child's body. Accordingly, the symptoms may return again and again (it seems, rather, that they do not disappear completely) so long as such levels of lead concentration persist within the body.

Chelation therapy designed for the effective elimination of lead. In making the most effective use of $CaNa_2EDTA$ or of other chelating agents with similar characteristics, attention should be given to means of determining when the body burden of lead has been reduced to a suitable level. This need not be a matter of conjecture. The pattern of the metabolism of lead is now sufficiently well known, descriptively, even after it has been distorted repeatedly by therapy, to serve as a guide to this end. A brief review of the facts will serve to illustrate the principles involved.

The absorption and excretion of lead, under the usual or "normal" conditions of North American life, occur within well-defined limits which have been described in detail. Under these conditions, lead is found within reasonably well-defined limits in all the tissues of the body, much the largest quantity, as well as the highest concentration, being found in the skeleton. The level of concentration varies appreciably from person to person, but remains fairly constant, after early infancy, throughout life (2). When the absorption of lead is increased beyond the usual level, the quantities of lead in the tissues increase, and the rate of the excretion of lead increases, in proportion to the increase in the rate of absorption. The increase in the body burden of lead in the intact organism is best shown by the concentration of lead in the blood, but the increase in the rate of absorption is disclosed first by an increase in the rate of the urinary excretion. However, since the urinary excretion of lead is influenced appreciably by a number of physiological factors, in addition to the rate and extent of the absorption of lead, it is difficult, except under carefully controlled conditions, to interpret in quantitative relation to absorption. Quantitative determination of the lead content of the blood is, therefore, the procedure of choice in the appraisal of the body burden of lead (3). When the concentration of lead in the blood reaches a level of 80 micrograms per 100 grams, or slightly in excess of this level (with due allowance for analytical precision, approximately $\pm$ 10 per cent, under the best conditions), there is imminent danger of intoxication. Values significantly below that level are not associated with intoxication, while values above this threshold coincide with an increasing incidence and severity of intoxication, in rough proportion to the degree of elevation of the concentration (3).

Following the administration of $CaNa_2EDTA$ intravenously (or intramuscularly), the concentration of lead in the blood may increase significantly (apparently this does not always occur), and the rate of the urinary excretion of lead increases after a time and continues for some hours, thereafter. The repetition of the dose or, better, the continuation of the intravenous infusion of $CaNa_2EDTA$ for several hours per day on each of several successive days, will result in a variable but persistent degree of elevation of the urinary excretion of lead during, and for a day, more or less, after, the period of administration. At this time, the concentration of lead in the blood will have diminished, perhaps to a low, essentially normal level; more commonly, the concentration in the blood will be appreciably but not strikingly lower than it was initially; it may not be below the threshold of danger. In general, the overall increase in the urinary output of lead varies directly with the quantity of lead available in the body for excretion (in response to equivalent dosages of the chelating agent), while the degree of change in the concentration of lead in the blood varies inversely therewith. If the concentration in the blood remains dangerously high, the therapeutic procedure should be repeated after an interval of several days; and this procedure should be repeated again and again until the concentration of lead in the blood has been brought within the normal range (under 60 micrograms per 100 grams or lower).

If now, the therapeutic task is taken to be complete, a serious error may result. For with the elimination of much of the lead from the soft tissues without a corresponding diminution of the lead content of the skeleton, the loss of lead from the skeleton, dependent upon its burden, will again elevate the lead in the soft tissues, including the blood, and concentrations almost as high as those found initially may be found again in the blood at the end of three or four weeks. Under such circumstances the chelation therapy should be resumed, if there are no contraindications, and the process should be repeated until the concentration of lead in the blood has been reduced and *maintained* within the normal range. It is to be remembered in this connection that the quantities of lead in the body at the onset of intoxication (except in rare instances in which the rate of absorption

of lead has been very great for only a few days or weeks) are likely to be large; in the adult, under occupational conditions, the quantity will usually be in excess of 1 gram, while in the small child, after some months of excessive absorption, one hundred to several hundred milligrams will have been absorbed. The loss of lead in the urine during several days of chelation therapy may be an insignificant proportion of that available for excretion. If such therapy is to be more than a gesture, it must be pursued to some rational endpoint. There seems no present means of determining when such a point has been reached without a precise and persistent analytical control of the process. Surely, in a situation in which the clinical response of the patient, even while under medical observation, is not a satisfactory criterion of the adequacy of the therapy, and in which so much faith is put in the beneficent effects of the elimination of the offending element from the body, every reasonable effort should be made to ascertain the extent to which this has been achieved.

REFERENCES

1. Chisolm, J. J., Jr. and H. E. Harrison. *Pediatrics* 19: 2, 1957.
2. Kehoe, R. A. *Occup. Med.* 3: 156, 1947.
3. Kehoe, R. A. *Indust. Med.* 20: 253, 1951.
4. Kehoe, R. A. In: *A Textbook of Medicine*, edited by R. L. Cecil and R. F. Loeb. Philadelphia: Saunders, 1959, p. 498.
5. Smith, H. D. *Indust. Med.* 28: 148, 1959.

DISCUSSION

Clinical experience with the use of calcium disodium ethylenediaminetetraacetate in the therapy of lead poisoning

HARRIET L. HARDY
Massachusetts General Hospital, Boston, Massachusetts

It is a privilege to participate even slightly in a forward looking enterprise such as this meeting in which students of a fascinating, relatively new field of knowledge attempt to communicate and pool their findings in spite of great differences in training and experience. This well recognized difference in training and experience is especially notable for the clinician like myself who works with the diagnosis, natural history, and therapy of disease. Data from patients are notoriously hard to control and lack the repeatability of the studies of those of you working in the more exact disciplines of the biological sciences, chemistry, and physics.

These remarks are introductory to presentation of the experience of my associates and myself with occupational metal intoxication in adults. My best contribution is, I believe, to report to you the questions that arise in my mind from clinical observations. These questions against the background of theoretical and experimental knowledge of chelating agents may provide clues that we clinicians need for improving our use of these drugs. In addition, such questions posed by the course of a patient under treatment may provide ideas for the direction of laboratory studies. My discussion is based on experience with the use of monocalcium disodium ethylenediaminetetraacetate ($CaNa_2EDTA$) in adults suffering lead poisoning of man-made origin. The diagnosis in these cases rests on documentation of the exposure to fumes of inorganic lead compounds of known toxicity confirmed by levels of lead and coproporphyrin in the urine and hematologic abnormalities generally accepted as correlated with disease.

Although the metabolism of lead is well studied it is, as far as I know at present, impossible to learn the exact body burden of the metal except under experimentally determined conditions. It is also not entirely known what rules of the body govern the amount of lead in soft tissue and what ratio this quantity bears to that in the skeleton at any given time. Kehoe, Foreman, and Schubert undoubtedly can offer data on this point. The rate of exposure, total exposure, and route of entry of the lead are among the factors that affect the burden of lead that must be dealt with in any given case with and

without clinically manifest intoxication. This problem of the size and location of the lead burden appeals to me as vital to understanding the merits and limitations of chelation therapy. It has been generally thought, on the basis of Aub's work, that lead in the body behaves as calcium does, and for this reason many variables are introduced which make clinical observations difficult to interpret. Aub (1) recently pointed out the obvious fact that abnormal states of calcium metabolism as, for example, in Paget's disease and hyperthyroidism result in the release of stored lead with calcium and greatly influence the clinical course of lead poisoning and the effect of a chelating agent.

One striking fact of my experience in the use of $CaNa_2EDTA$ in adult cases of lead intoxication has been that the quantity of lead removed varies from case to case with the same dose of the drug (Table 1). Perhaps this is not an unexpected finding, which you well understand. As I have suggested, size and location of lead body burden, route, the rate of entry into the body, and unrecognized complications such as infection or underlying renal disease may influence these findings. In addition, speed of administration of intravenous medication, and variations in calcium metabolism not only in certain diseases but under such physiological changes as aging, are all possible factors in the result of chelation therapy both in the relief of signs and symptoms and in the increase in excretion of lead.

I want to repeat that short term, high level exposures to lead are apparently handled by the body very differently than long term exposures at lower levels which are, however, high enough to overwhelm in time the body's resources for excretion and safe storage of toxic material—facts which may help to explain these variable findings.

In a few of the cases of industrial lead poisoning that I have had the opportunity to study well (9), I have been struck by the variation in the time that lapses after the use of $CaNa_2EDTA$ and the return to normal of such indices of intoxication as the hematologic abnormalities and the urinary coproporphyrin levels (Table 2). These data pose questions to me which you may be able to answer. Size and location of the lead body burden at the time of onset of manifest poisoning, dose of $CaNa_2EDTA$ administered, and causes other than lead poisoning for iron deficiency or increased coproporphyrin excretion complicate understanding of clinical observations. Knowledge of the effect of such materials as $CaNa_2EDTA$ on metals vital to enzyme systems, as the papers given at this meeting demonstrate, is growing and changing rapidly. It is therefore premature to state what will finally be known, for example, about the action of these agents on the iron, freed from the erythrocytes by the toxic action of lead. As I read and try to understand the literature in this field, it appears that iron and hemoglobin precursors may be bound by chelators under certain conditions. A problem in reversing the lead induced anemia thus may be created. Without the use of chelating agents, lead induced anemia and elevated coproporphyrin levels will spontaneously reverse if lead exposure is stopped. Could it be that that portion of the administered dose of $CaNa_2EDTA$ unused in chelating lead is entering into other complexing actions with results that explain these variable findings?

TABLE 1. *Amount of Pb Removed by $CaNa_2EDTA$*

	Dose, gm	Pb in Urine, mg/liter	Pb in Urine in 24 hr.
M. J.	4	17.80	13.00
M. M.	4	11.70	16.38
C. F.	3.5	1.27	2.50
S. E.	3	8.10	15.71
M. R.	2	3.60	8.80
McK. J.	2	2.64	6.30

TABLE 2. *Variation in Time Needed for $CaNa_2EDTA$ Effect*

	Duration of Treatment, days	Reversal of Hematologic Abnormalities	Urinary Coproporphyrin to Normal, days
M. J.	5	3 weeks	10
M. M.	4	5–8 weeks	18
C. F.	5	7 days	4
McK. J.	7	7 days	5
S. E.	6	6 days	4
W. S.	24 (18 + 6)	6 months	6
M. R.	15 (3 × 5)	5 weeks + (no follow-up)	18

Those of you who understand the equilibria of the action of chelating agents, doubtless find no trouble explaining the fact that continuous doses of $CaNa_2EDTA$ administered on successive days result in decreasing levels of urinary excretion of lead. It seems curious to me that the lead located in the parts of the skeleton where it may be mobilized into the soft tissue does not move in when those depots are empty unless the drug is stopped. In practice, intravenous administration of $CaNa_2EDTA$ is usually given as slowly as patient and hospital staff will tolerate, taking from three to six hours. Since the action of the chelator is at its height within three to five hours and falls off rapidly with complete excretion of the drug, I (undoubtedly from ignorance) fail to understand why skeletal lead does not come into soft tissue during the eighteen hours of rest from chelation administration. Perhaps enough EDTA remains in the body to prevent such redistribution.

A very practical matter that some of you may find interesting to consider, and with which those like myself who treat patients need help, is the problem of how long to continue deleading by chelation. We do not know the total body burden of lead; but, based on reasonably complete knowledge of the size of the dose workers may inhale in lead using operations, the amount of lead taken from the body by a short course of $CaNa_2EDTA$ is a token quantity only. Even with no further lead exposure, it is my experience that workers who have had long periods of toxic lead inhalation and one or more episodes of acute poisoning will suffer, without further lead intake, exacerbation of signs and symptoms apparently precipitated by some disturbing event in body metabolism which our methods of detection are often too unsophisticated to describe. Should the clini-

cian treat at intervals again and again with a short course of the chelator as required by signs and symptoms, or should he in the first place have insisted on more deleading?

Those who treat childhood plumbism must continue to delead until it is quite certain that the body burden of lead is small enough to preclude later mobilization with damage to the central nervous system. In adults, lead neuropathy is certainly indication for repeated courses of chelation therapy. This is especially true since Bell's (3) report of a case of extensor palsy of twenty years' duration in a lead worker which was reversed by one and one-half years of intermittent $CaNa_2EDTA$ treatment. Reporting this case informally, Bell brings out a finding we also noted, that the lead urine levels do not correlate in all cases with lead damage after intermittent, low level lead exposure of long duration.

A case of ours illustrates this point—a man with long term but intermittent lead exposure resulting in illness requiring eleven hospital admissions and a wide variety of diagnoses until he suffered bilateral wrist drop (Table 3). With $CaNa_2EDTA$ treatment, the extensor palsy disappeared and he was able to return to his job as a painter within four months. It is only fair to mention that without lead exposure extensor palsy will spontaneously reverse in time in most cases but it is the current feeling that $CaNa_2EDTA$ therapy has improved the prospect for early return of function as well as offering benefit to lead induced palsy of long duration. H. W. Moser (personal communication, 1960), at the Massachusetts General Hospital, treated an unusual case of lead neuropathy with $CaNa_2EDTA$ alternating with calcium trisodium diethylenetriaminepentaacetate ($CaNa_3DTPA$), with improvement which at first seemed slow; but within six months the patient was able to work. The $CaNa_2EDTA$ resulted in a strikingly higher urinary lead level than did the DTPA. I wonder (others have, too) whether high excretion levels are the essential criterion of beneficial effect of $CaNa_2EDTA$ treatment in lead poisoning.

Discussing practical problems in using chelating agents brings up the question of possible harm of using $CaNa_2EDTA$. The observation of H. D. Smith (personal communication, 1958) and others that, at the outset of treatment of acute plumbism in children with intravenous $CaNa_2EDTA$, there is often an exacerbation of cerebral symptoms certainly requires explanation. Smith suggests that lead EDTA may not be as stable a complex as thought. When I asked Teisinger of Prague, who has had experience and was recently here, his opinion of this clinical experience, he proposed that in a child the dose not only of lead but lead EDTA is relatively great; and, in addition, the lead EDTA complex may not be completely harmless to growing tissue.

In adult patients, we have had no systemic reactions like those described by Seven (11), and in only two cases have we had complaints hard to explain, close in time to the onset of chelating therapy. W. H. Baker (personal communication, 1960), at the Massachusetts General Hospital, made an interesting observation on one of our well studied cases of industrial lead poisoning. He alkalinized the urine with Diamox and then gave $CaNa_2EDTA$ by vein. The patient suffered abdominal colic after the first infusion. Baker suggested that the man had true lead colic as a result of this maneuver. You doubtless have knowledge with which to agree or debate this conclusion.

TABLE 3. *Lead Found*

Date	Coprecipitation Method mg/liter*	Ashing Method mg/liter*	
F. C. 8/28	0.10		
8/30	0.07		
8/31	0.10		
9/1	0.08		
9/2		1.17	Drug
9/3		1.61	Drug
9/4		2.10	Drug
9/5		1.76	Drug
9/6		1.32	Drug
9/7		1.75	Drug
9/8		1.13	Drug
9/9	0.01	0.56	
9/10	0.04	0.13	

* Adjusted to 1.024.

Failure of the coproporphyrin excretion to return promptly to normal levels and hematologic changes to reverse for variable periods of time plus, in some cases, continuation of symptoms consistent with toxic lead effect suggest that lead in blood may be increased at times during therapy with $CaNa_2EDTA$ under certain circumstances. Seven (11), Foreman (7), and others have discussed this possibility and the experience of Byers (6) and Smith (personal communication) and Kehoe (personal communication) with childhood plumbism provides supporting evidence. Further, according to Bessman and Layne (4), there is an increase in the shift of lead from the erythrocytes to plasma during the five hours following a three hour infusion of $CaNa_2EDTA$. Putting the available evidence together, the clinician appears to be taking a certain amount of risk using $CaNa_2EDTA$ for lead poisoning especially in children. In practice, however, during the past seven years of use in adult lead intoxication, no harm has been documented in our series.

The evidence that oral $CaNa_2EDTA$ is harmful seems incontrovertible. Byers' (6) report of brain damage in a child with nonsymptomatic plumbism given two .5 gm tablets of $CaNa_2EDTA$ six hours apart is sobering evidence. Rieders (10) states that the lead chelate goes through the intestinal wall after which lead levels in liver and brain go up. Yet Foreman (8) and others report very poor absorption of such compounds through

TABLE 4. *Lead Found*

Material	Weight, gm	mg Pb/Sample
Humerus cortex	9.2	0.898
Humerus medulla	5.7	0.483
Kidney	6.2	0.003

the intestinal wall. Recently, I have been shown data from a well controlled series of asymptomatic ambulatory lead workers, exposed to above so-called safe levels, with consistently elevated urinary lead levels after oral $CaNa_2EDTA$ considered well above what would be supplied by lead to be found in the feces, with coproporphyrin levels returning to normal. Agreeing that oral $CaNa_2EDTA$ for therapy of lead absorption is unwise for many reasons, it seems to me the knowledge available at present to explain the behavior of the orally administered drug is completely confusing.

In the cases I have treated with $CaNa_2EDTA$, I have seen no evidence of renal damage. Discussing this problem with colleagues from abroad, I find there is considerable disagreement as to the evidence for renal damage (2, 5) caused by $CaNa_2EDTA$ used in the accepted safe dose range. Some evidence is provided by my colleague, Baker, at the Massachusetts General Hospital who carried out metabolic studies, one of eighteen consecutive days of treatment and one of six days in two workers with chronic lead poisoning. Careful renal function studies before and after treatment showed no evidence of harmful effect of the drug on the kidney. Balance studies of calcium, phosphate, nitrogen, potassium, sodium, chloride and magnesium showed no change. These studies are to be published in full.

Also of interest is the case of a structural steel painter with at least three well documented attacks of acute lead effect and treatment with $CaNa_2EDTA$ intravenously and orally, intermittently for three years, as seemed required clinically, who came to postmortem study recently. He had shown albuminuria consistently for two years before his death. At autopsy he proved to have pulmonary malignancy with metastases involving his kidneys. Careful examination of the kidney by Dudley showed no evidence of harmful effect of the chelating agent. Table 4 shows the levels of lead in the humerus and kidney in this same worker at his death, approximately five years after the last date he might have had the slightest lead exposure. The patient had received a short course of $CaNa_2EDTA$ by mouth one year before his death following intravenous therapy on four occasions during the fifth, sixth, and seventh years before his death. It is of interest to read you Dr. Kehoe's report on these findings, which he points out is limited by the lack of full data on dates and extent of lead exposure. I quote: "The similarity of the levels of lead concentration in the two parts of the humerus indicates that a long time (at least some months, and probably some years, dependent upon the severity and duration of the exposure) had elapsed between this man's death and the termination of his exposure.

"The concentration of lead in the humerus is considerably higher than normal (approximately 6 to 7 times the normal level) but not nearly so high as it is found to be, frequently. This too speaks for a long interval of freedom from occupational exposure, although this may have been influenced (appreciably but not drastically) indirectly by the chelating therapy employed.

"The concentration of lead in the kidney is quite low (about one-fourth of the mean normal value) which may be a matter of chance or of therapy, dependent upon the interval of time between the last course of therapy and death."

In summary, then, as a clinican, I am impressed with the possibilities of therapy with presently available chelating agents. Prompt reversal of hematologic abnormalities; return of coproporphyrin levels to normal; relief of neuropathy; and measurable improvement in the prognosis of childhood plumbism are real and objective criteria of clinical effect of $CaNa_2EDTA$ in treatment of lead poisoning. There are gaps in knowledge which appear as questions in the mind of the clinical observer, but there appears clearly the prospect of helping without harming certain patients with heavy metal poisoning. Further, the use of chelating agents promises to develop new knowledge of the mechanism of metal intoxication.

REFERENCES

1. Aub, J. C. Discussion, The Lead Hygiene Conference, Chicago, November 6–7, 1958, *Indust. Med. and Surg.* 28: 41, March, 1959.
2. Belknap, E. L. Review of Ten Years' Published Experiences of Treatment of Inorganic Lead Absorption in the Adult with Edathamil or Calcium Sodium EDTA. The 13th International Congress on Occupational Health, New York City, N. Y. July 25–29, 1960.
3. Bell, R. F. Discussion, The Lead Hygiene Conference, Chicago, November 6–7, 1958, *Indust. Med. and Surg.* 28: 77, March, 1959.
4. Bessman, S. P. and E. C. Layne, Jr. *A.M.A. Am. J. Dis. Child.* 89: 292, 1955.
5. Brugsch, H. G. *A.M.A. Arch. Indust. Health* 20: 285, 1959.
6. Byers, R. Discussion, The Lead Hygiene Conference, Chicago, November 6–7, 1958, *Indust. Med. and Surg.* 28: 61, March, 1959.
7. Foreman, H., C. Finnegan and C. C. Lushbaugh. *J.A.M.A.* 160: 1042, 1956.
8. Foreman, H. and T. T. Trujillo. *J. Lab. & Clin. Med.* 43: 566, 1954.
9. Hardy, H. L., H. B. Elkins, B. P. W. Ruotolo, J. Quinby and W. H. Baker. *J.A.M.A.* 154: 1171, 1954.
10. Rieders, F., W. G. Dunnington and H. Breiger. *Indust. Med.* 24: 195, 1955.
11. Seven, M. J. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 95.

GENERAL DISCUSSION

DR. PETERS: I have enjoyed the last three presentations very much and would like to comment relative to Dr. Hardy's presentation in which a patient was described who began to develop more severe symptoms after being given EDTA plus Diamox which, as you know, is a sulfonamide derivative.

Cecil Watson, Waldenstrom and others, including ourselves, have been impressed by the similarity of some cases of acute porphyria to cases of lead intoxication. The porphyric patients almost invariably are made worse by the administration of sulfa drugs, and in both the porphyric patients and in lead intoxication there is an increase in urinary coproporphyrin III excretion. As a matter of fact, when the coproporphyrin excretion becomes elevated in patients exposed to lead, they usually are beginning to develop signs of lead intoxication.

I would like to suggest that possibly the reason this patient given EDTA plus Diamox got into more trouble was because Diamox, a sulfa drug, further interfered with this patient's porphyrin metabolism. Possibly in some of these cases of childhood lead intoxication, the pediatricians become too diligent in the treatment of seizure or epileptic phenomenon and employ barbiturates for the control of these seizures which further interferes with their porphyrin metabolism, thus making their lead intoxication worse. I would like to recommend in the child with lead intoxication that barbiturates and sulfa drugs be avoided, and other drugs such as paraldehyde be used for seizure control.

I should like to comment on the neurosurgical decompression that sometimes becomes necessary in cases being treated for lead intoxication to prevent fatal brain swelling. Doctors Javid and Settlage of the University of Wisconsin introduced the use of urea in the treatment of cerebral edema as seen in brain tumors, for example, and to promote shrinkage of the brain to give better exposure during brain surgery. Dr. Richard A. Katz has reported in the April 28, 1960, New England Journal of Medicine on the use of intravenous urea in the therapy of increased intracranial pressure incidental to lead encephalopathy. It would seem to me that this might be a rational alternative to more extensive and mutilating neurosurgical decompression.

Finally, I would like to raise the question as to the advisability of adding BAL to the therapy of some of these lead intoxication patients that do not do well when treated with EDTA alone. Although BAL itself would seem to be contraindicated to be used alone, at least in the initial treatment of lead intoxication, some people feel that sulfhydryl groups are capable of imparting greater stability to the lead chelate, and, at least in our porphyric patients, we have frequently interchanged BAL and EDTA, sometimes with better response than when we have used one or the other agents alone.

DR. SCHUBERT: I have a few comments about the treatment of lead poisoning. I think that in years to come the use of chelating agents—that is, the readily excretable types such as EDTA—for treatment of nonradioactive metal poisons will rarely be employed. I think it is potentially a dangerous method, especially if the delayed effects are considered. Nonradioactive metals do not have to be removed from the body in order to inactivate them so they can't react with tissues.

The dangers of treatment with chelating agents are quite obvious and can be shown experimentally (Schubert, J. In: *Chemical Specificity in Biological Interactions*, edited by F. R. N. Gurd, New York: Acad. Press, 1954, p. 114, and Schubert, J. and M. W. Rosenthal. *A.M.A. Arch. Indust. Health* 19: 169, 1959.). For example—the total toxicity of a metal is not a function of its total concentration in the body but only of that fraction which can react with tissues. In other words—if you administer lead as an insoluble sulfate, you find that it is less toxic than the more soluble lead salts. When you give a chelating agent such as EDTA, you enhance the solubility of the lead, i.e., you form lead chelate, causing it to be transported around the body. Depending on local conditions, the concentration of the chelating agents and the stability constant, some of the lead chelate will dissociate, thus transporting lead ions to tissues where lead normally doesn't penetrate. We have been able, experimentally, in the past several years, to modify the toxicity and distribution of many metals at will simply by varying the ratio of chelating agent to metal. In some cases the toxicity of metals is greater than without use of a chelating agent.

The behavior of lead illustrates some interesting points. If lead is administered carrier-free it remains for a long period in the blood without disappearing (Schubert, J. and M. R. White. *J. Lab. & Clin. Med.* 39: 260, 1952.). However, if weighable amounts of lead are given it disappears very slowly until the red blood cells are saturated—then the excess spills over into plasma and that fraction disappears rapidly. The plasma lead is distributed rapidly to the tissues while the amount in the red blood cells is held for much longer periods, released slowly, and presumably can be better tolerated by the body. Therefore, when treatment causes a big increase in lead excretion, it is because the plasma lead and the more accessible fractions are chelated but not the lead bound to the red blood cells or deposited within the bone.

In order to evaluate the potential effectiveness of different chelating agents, one must take into account competition from other ions in the body. The relative effectiveness of two chelating agents, for example, are best compared in *in vitro* systems when the solutions approximate the physiological composition. Simple ultrafiltration studies on lead (Fried, J. F., J. Schubert and A. Lindenbaum. *A.M.A. Arch. Indust. Health* 20: 473, 1959.) with DTPA (diethylenetriaminepentaacetic acid) and BAETA (2:2′-bis(dicarboxymethyl)aminodiethyl ether) have shown that in the absence of plasma protein, equimolar amounts of DTPA or BAETA were equally effective as far as increasing the diffusibility of lead is concerned. However, as soon as plasma was added to the test system, the percentage of Pb which was ultrafilterable (80–90 per cent) dropped to 11% with BAETA but remained high with DTPA.

When a chelating agent is administered it is rapidly excreted; however, the diffusibility or availability of the lead within the tissues is rendered very high by even small amounts of many chelating agents. Since the tissue sites serve as a storage depot for lead, the increased diffusibility of the lead chelated *within* the tissues (Lindenbaum, A. and J. Schubert. *Nature, London* 187: 575, 1960.) causes the release of lead to

the blood to proceed at a rate higher than would be the case if no treatment were given. Consequently, I think that the treatment of choice in the future will be lake-forming dye stuffs rather than the diffusible chelating agents. However whenever I bring up the idea of using materials which do not cause a change in the excretion pattern of lead or beryllium or other toxic nonradioactive metals, the first question I get is "How much lead or beryllium do you get out of the body?" When I answer "None," the questioner seems surprised.

We have demonstrated that with beryllium the use of lake-forming dye stuffs causes no appreciable change of excretion (Schubert, J. and M. W. Rosenthal. *A.M.A. Arch. Indust. Health* 19: 169, 1959.). However, the dye has a distribution similar to the metal as shown by radioautograph and histological studies, and the beryllium is inactivated within the tissue preventing further cell damage. Any damage which has occurred becomes repaired, and no further damage occurs during the life of an animal. It must be appreciated that treatment with ordinary chelating agents only removes a small fraction of the total metal in the body. So at best the patient must live with most of the metal—hence why not render these permanent deposits less soluble?

I believe the treatment of choice for lead and other occupational metal poisons eventually will be the use of chelating dye stuffs of which there are hundreds of possibilities. For lead the obvious type of chelate lake-forming dye would be a so-called vat dye or other soluble dye with sulfhydryl groups in proper position. There are numerous such dyes that you can use and test for treatment. I want to emphasize my belief that *excretable* chelating agents are potentially dangerous for treatment. There are patients known to have died shortly after injection of EDTA or BAL. I think such unfortunate incidents reflect an enhanced penetration of lead into the blood brain barrier and other susceptible tissues as a result of the increased availability of toxic metal ions induced by the chelating agents.

Dr. Aposhian: Why hasn't penicillamine been studied more extensively in the treatment of intoxication by inorganic lead compounds? There are only one or two reports in the literature.

Dr. Paul Hammond (University of Minnesota, Minneapolis): By way of introduction, I might say that the problem of lead poisoning in cattle is a very real one. We do see the condition quite frequently and it resembles the lead poisoning in children more closely than it does that in adults, in that it is primarily an encephalopathic disease. We reported results on attempted comparisons of various chelating agents with regard to excretion of lead in cattle at the New York Academy of Sciences meeting that was held last December. However, I would like to overlap a little bit on that information and repeat one thing, and perhaps add a couple more things that I think are germane to the present discussion, particularly the concern over the toxicity of the EDTA lead chelate.

We administer lead to calves and we get a fairly reproducible response to the EDTA in terms of the excretion of lead in urine and in terms of the plasma curve. If we administer EDTA at zero time to a calf we get a mobilization of lead into the plasma which follows an exponential form and tapers off back to the near zero point at about 12 to 18 hours. Now, accompanying this is an excretion of lead in urine—the magnitude of which depends largely upon the concentration of lead in the red blood cells at the time that the experiment is initiated. We have compared a number of chelating agents using this preparation. Penicillamine does produce a very appreciable increase in the urinary excretion of lead which is none the less inferior to that of EDTA. I don't say that on the average

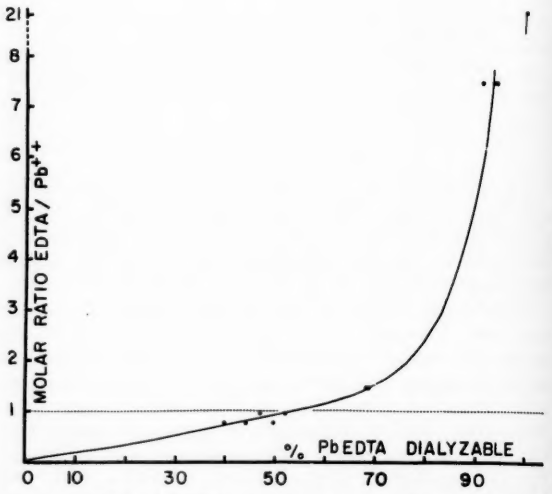


FIG. 1. Effect of EDTA concentration on the diffusion of lead from bovine plasma.

penicillamine would prove inferior to EDTA. The one animal in which we tested this did give a response which appeared to be inferior to the average result obtained with EDTA.

I was talking to Dr. Aposhian about another observation concerning the use of N-acetyl-penicillamine. We administered this drug and obtained no rise in the concentration of lead in the plasma until approximately two hours following the administration of the drug. Now this is highly anomalous. In all other cases, the mobilization of lead in the plasma is almost instantaneous and does follow this rapid decline. In the case of N-acetyl-penicillamine we got a small rise in the concentration of lead in plasma at about two hours and a return to normal within two hours following this. (Incidentally, this is accompanied by an increase in excretion of lead in the urine of a very minor magnitude and also one that is very transient). We were wondering if perhaps it might not be a reflection of some metabolic process in the body whereby the N-acetyl-penicillamine is deacetylated to form penicillamine. May I note one more thing here? Dr. Schubert mentioned results obtained concerning the binding of DTPA lead in plasma. We've done this with EDTA and obtained results as shown in Figure 1. In plotting the molar ratio of EDTA to lead, when the molar ratio was one, we got about 50 per cent binding. Only at a molar ratio of about 20 to 1 did we obtain no detectable lead in the dialysate. These results suggest that, regardless of how high the stability constant may be for the lead EDTA complex, under the conditions of body pH, in competition with the calcium ion and perhaps others in the body, you none the less perhaps have to maintain a rather high covering level of EDTA in the blood in order to insure no dissociation of the lead that has been mobilized.

Dr. Eichhorn: I would like to point out that not only don't we chemists have all the answers to questions biologists ask, sometimes we have questions to ask of the biologists. I was intrigued by Dr. Kehoe's statement that apparently it is possible to eliminate lead poisoning by the lapse of time. Those of us who are in chemistry laboratories are concerned somewhat about mercury poisoning and some of us have the understanding that it is pretty difficult to get rid of this. I wonder if someone has a comment to make about the analogy with mercury poisoning.

DR. KEHOE: In brief answer to Dr. Eichhorn's question, I would say that mercury is not retained in the tissues as is lead. Generally speaking, mercury is eliminated fairly rapidly from the tissues. The problem is not so much that mercury is *retained* over a long period of time after illness is discovered, but rather that it has been *absorbed* over a very considerable period of time before illness is noted, and, therefore, the damage has had time to be cumulative. The problem in the case of mercury is that of irreversible damage, particularly to the brain. Here the problem is certainly one of prevention. If you keep the concentration of mercury in the atmosphere of the laboratory low enough, there will be no mercury poisoning.

DR. ROBERT F. BELL (University of Colorado): A few remarks on some preliminary observations we have made on the study of the long term excretion of tagged lead in dogs and its enhancement by EDTA may be pertinent at this time. We gave dogs a single intravenous dose of carrier-free radioactive Pb^{210} and followed its excretion. The fecal Pb^{210} excretion was found to be less than one hundredth of the urinary excretion and is disregarded in our study of total lead excretion. Our dogs excreted 2.5 to 3 per cent of the amount given during the first day. After the first day the daily rate of excretion based only on partial data has approximated $y = 0.0066t^{-0.896}$ where y is the fraction of the original dose and t is in days.

DR. HARRY FOREMAN: What then is the biological half-life of lead?

DR. BELL: A primary purpose of our study is to determine the biological half-life of lead but our results defeat our purpose since it is not possible to express a biological half-life when the excretion follows a power rather than the expected exponential function. Norris *et al.* (Norris, W. P., S. A. Tyler and A. M. Brues. *Science* 128: 456, 1958.) have reported similar observations in their study of other radioactive bone seekers. Your question may be satisfied partially by the calculated cumulative per cent excretion per time. The first day 2.67 per cent is excreted and in the first year 8.03 per cent. After ten years 11.24 per cent would be excreted and in one hundred years 15.24 per cent. Approximately 8.057×10^8 or nearly a billion years would elapse before 50 per cent was excreted. The effective biological half-life of radioactive Pb^{210} on the other hand is determined by its physical half-life of 22.2 years and by our calculations would be somewhat over 17 years.

While natural excretion of lead is a power function and cannot be expressed by a half-life, the excretion of EDTA and the lead EDTA complex are exponential functions and are expressed by and compared by their respective half-lives. Foreman (Foreman, H. and T. T. Trujillo. *J. Lab. & Clin. Med.* 43: 566, 1954.) found the EDTA human biological half-life as 60 minutes; we found the dog biological half-life as 50 minutes. We found the lead enhanced by EDTA human half-life as 300 minutes (*Proceedings of the Lead Hygiene Conference*, Lead Industries Association, Chicago, Ill., November 6–7, 1958, p. 69 or Bell, R. F. *Indust. Med.* 28: 153, 1959.) and the dog biological half-life as 200 minutes. We also found the tagged lead EDTA complex dog half-life as 50 minutes. This strongly suggests that the lead EDTA complex is not dissociated in its passage through the blood or kidneys.

A 3 gm human equivalent intravenous dose of EDTA given one to three years after the original dose of lead provoked an excretion of 0.15% of the calculated remaining body burden of tagged lead. Under our experimental conditions, the provocative test appears promising but it needs further study.

The comments here tonight demand some further elucidation of the case of lead neuropathy for twenty years briefly alluded to in our remarks in a discussion at Chicago (*Proceedings of the Lead Hygiene Conference*, Lead Industries Association, Chicago, Ill., November 6–7, 1958, p. 69 or Bell, R. F. *Indust. Med.* 28: 153, 1959.). After four years on a high lead exposure job as a furnace and roaster man in a lead smelter, the patient developed a severe bilateral wrist drop and extensor finger weakness. Because of his severe lead neuropathy, he was placed on what was then and is today considered a non-lead exposure job in raw ore. He remained out of lead exposure on this job until we saw him over nineteen years later. He told us that he was unable to open his hands at all when he was taken off the high lead exposure job but, after some fifteen shots of what we assume was intravenous calcium and two years after he was out of lead exposure, he stated that he could open his hands slightly. This neuropathy did not improve further until after we had seen him. We made a diagnosis of a lead neuropathy from his history of onset during a heavy lead exposure and from his clinical signs in spite of the fact that his urinary lead excretion was only 0.08 mg per 24 hours.

We were studying provocative EDTA excretions at that time and out of curiosity as to how much lead this man might excrete with his history of exposure and neurological signs rather than any thought of treatment, we gave this man 3 gm of EDTA intravenously and were rather surprised on the following day to find his neurological picture improved. After receiving a report of 5.64 mg per 24 hours enhancement of lead by our trial of EDTA, we thought that our noted slight clinical improvement might have been due to the EDTA and decided to give him a therapeutic trial of EDTA. On intermittent intravenous EDTA therapy his muscular weakness rapidly improved for two to three months and then improved rather slowly for the next two years. He returned to his former job after four months of treatment and has remained there ever since. We saw this man recently five years after he had completed his EDTA treatment and over six years after he had returned to his former raw ore job. There was no evidence of recurrent peripheral neuropathy. His urinary lead excretion was 0.05 mg per 24 hours and 3 gm intravenous EDTA provoked 3.15 mg excretion.

We apparently all agree that in humans certain clinical signs and symptoms are associated with high lead exposures and presumably with high lead absorption. Some of these signs and symptoms are associated with increased blood and urine lead concentrations but others are not. Our preliminary dog data suggest that only an insignificant amount of absorbed lead normally is excreted. Dr. Hardy has been impressed and we also have been impressed by the remarkable clinical improvement after only a token removal of lead with EDTA. We suggest that the body or metabolic location of lead is the cause of clinical signs of lead poisoning and that this location is determined by age, individual and varying metabolic patterns, quality as well as quantity of lead exposure and the body burden of lead. Does it seem unreasonable to assume that a strong chelating agent such as EDTA might dislodge a small quantity of lead from some critical metabolic site where our normal body metabolism might not? Is it then unreasonable to expect EDTA to bring about rapid clinical improvement in lead poisoning?

Radioactive metal mobilization

A. CATSCH

Institute of Radiobiology, Nuclear Research Center, Karlsruhe, Germany

THE BIOLOGICAL EFFECTS of carrier-free radionuclides absorbed by the living organism are, with very few exceptions, a consequence of the ionizing radiation emitted. Accordingly, the therapeutic aim in cases of poisoning with metals will differ according to whether the metals are or are not radioactive. If they are not, the conversion of the metal into an insoluble and thus pharmacologically inactive form or a change in its distribution pattern can sometimes be taken as a therapeutically satisfactory result; if on the other hand the metals absorbed are radioactive then the sole aim of the therapeutic measures taken must be their removal from the organism. More precisely, the aim is to reduce the radiometal concentration primarily in the critical organ, that is to say the organ in which injury due to deposited radioactivity would be most serious for the organism. We may say here that as far as most radiometals of practical interest or potential hazards are concerned, the critical organ is the skeleton.

When the problem of radioactive poisoning and its treatment first aroused attention in view of the increasing production and use of radioactive substances, no known complexing agent, or none at any rate that had been tested biologically, had the qualities required to make a substance effective: namely, a sufficiently broad therapeutic range, inert behavior in metabolism, and, above all, a high affinity for the radiometals to be removed from the organism. Dimercaptopropanol (BAL), which had proved relatively effective in animal experiments and also in clinical tests in cases of poisoning with various stable heavy metals, failed generally against radiometals of practical interest (49, 50, 61), with the one exception of polonium-210 (43, 44). The first important advance was made with ethylenediaminetetraacetic acid (EDTA), which for a considerable time remained the only chelating agent which had been at all extensively tested on animals and also showed favorable and encouraging clinical results (28, 62, 64). The earlier work on EDTA has in recent years been thoroughly surveyed (2, 6, 17, 64, 67, 71), and there is no need to go over its results in detail here. However, it will be useful to outline briefly the potentialities, drawbacks and limitations of the EDTA treatment.

The activity spectrum of EDTA is rather restricted by the fact that, even under optimum conditions (of dosage and time of administration), it is ineffective or only partially effective against a number of radiometals of practical importance. It has more than once been established that EDTA cannot prevent the accumulation of radium, radiostrontium or radiobarium in the skeleton. Our own experience (14) shows that it actually causes a 10–20 per cent increase in deposition, a fact which is consistent with Spencer's observation (77) on the inhibition of radiostrontium excretion in the urine in man after treatment with EDTA. EDTA also proved totally ineffective with ruthenium-106 (78). The increased excretion of radioactive light lanthanides, such as cerium-144, is entirely attributable to a reduced deposition in the soft tissues, especially the liver; the fixation of cerium in the skeleton is either unaffected by EDTA or slightly increased (9, 10). Radioyttrium and plutonium respond fairly well to EDTA. But here we come upon the second disadvantage of EDTA: the marked dependence of its effectiveness on the time of administration. If EDTA is administered under conditions of delayed treatment, that is at a time when the major part of the radiometal has already been deposited in the tissues, the amount of radionuclide which can be mobilized is often small and without practical significance in relation to the remaining body burden. This is also true when the EDTA treatment is extended over prolonged periods, which in any case would seem to be a questionable procedure in view of the nephrotoxic action of EDTA.

These facts governed the direction and aim of subsequent investigations: first, the analysis of factors generally relevant for biological effectiveness, and, secondly, the search for other substances more effective than EDTA and/or having a broader therapeutic range. Of course these two problems are closely connected, since the prerequisite of a rational and systematic search for new pharmacologically effective compounds is a sound knowledge of their mode of action. It was early recognized (42, 67, 71) that the effectiveness of a chelating agent is decisively affected by competition between the metal ion to be removed and natural body cations for the chelating agent. Provided the concentration of chelating agent in the organism is high, competition by trace metals present only in low concentrations can be ignored and further considerations are restricted to Ca ions and in some cases hydrogen ions. Without going more deeply into the theory (42), this means that the effectiveness of a chelating agent Z in regard to the

mobilization of a radiometal M should be related in a first approximation to a quantity designated E:

$$E = \frac{K^{M}_{MZ} \cdot Z_{total}}{\alpha + K^{Ca}_{CaZ}(Ca)}$$

This holds good on the simplifying assumption that the radiometal is free from carrier, or at least has high specific activity, that the chelates of Z with biological trace metals can be ignored, and that at physiological pH only simple 1:1 Ca or M chelates are formed. The figure α designates the proportion of the free anion Z^{-i} in the total of all uncomplexed protonated species of the acid H_iZ and is calculated from the acidity constants and the given pH:

$$\alpha = 1 + K^{H}_{HZ}(H) + K^{H}_{HZ} K^{H}_{H_2Z}(H)^2 + K^{H}_{HZ} K^{H}_{H_2Z} K^{H}_{H_3Z}(H)^3 + \cdots .$$

If information is available on the stability of the hydrogen and hydroxo complexes, the numerator in E should be multiplied by the distribution function γ to obtain the total of all complexed M species; as a rule only slight and negligible variations of the E values are obtained as a result. Since at pH 7 for EDTA and its related polyamino acids α is considerably smaller than K^{Ca}_{CaZ}, the competition of hydrogen ions needs to be considered only at considerably lower pH values. Thus, for example, in acid urine a rather more marked breakdown of the M chelates and—if the metal ions are reabsorbed in the tubules—a loss of effectiveness are to be expected. Moreover, extremely low pH values are found in the secreting cells of the stomach. Dudley (20) attributed to a similar instability of the chelates the high concentrations of radioyttrium which he observed in the stomach wall after administration of the metal in chelated form.

If the effectiveness of a chelating agent with various radiometals is considered in relation to the K^{M}_{MZ}/K^{Ca}_{CaZ} values, a satisfactory correlation is generally not obtained. Figure 1 shows the effectiveness of EDTA expressed as the radiometal content of the organ after EDTA treatment as a percentage of the content of the organs of untreated control animals, for a number of carrier-free radionuclides under identical test conditions of dosage and time of administration of the EDTA. The lack of a clear relationship between the two variables was to be expected, because one relevant factor had hitherto been ignored, namely the competition between the therapeutic chelating agent and endogenous high-molecular chelating compounds (proteins, nucleic acids, etc.), ion-exchanging structures and, in some cases, hydroxyl or other anions for the radiometal. It is at present difficult to give even a semiquantitative treatment of these relationships because the appropriate acidity and stability constants are not yet known, nor is there sufficient information about the type of chelates involved. If a chelating agent is to have any effect on the biological behavior of a metal ion, then the pM defined by E

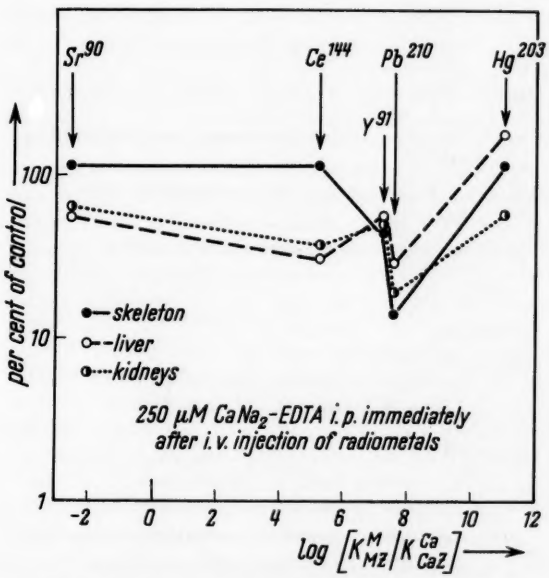
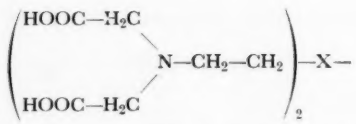


FIG. 1. Effect of EDTA under near optimal conditions on the retention of carrier-free radionuclides in the organs of the rat (Catsch *et al.* (10, 13, 14) and unpublished results).

must at least be as great as or greater than the pM determined solely by the biological milieu in the absence of Z, and the greater the difference between these two pM values the higher the effectiveness should be.

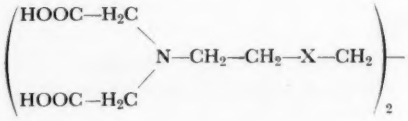
Since the concentration of competing endogenous ions and chelating agents and exchange capacity of the ion exchangers is nearly invariant and since the toxicity of the chelating agent makes it impossible to increase the dosage at will, the therapeutic effectiveness can—at this stage in the discussion—be improved only by varying the quotient K^{M}_{MZ}/K^{Ca}_{CaZ}, in other words, by the use of chelating agents which are significantly different in this respect from EDTA. There is no need to deal in detail here with the numerous chelating agents that have been examined during the past few years and proved inferior to EDTA. In order to emphasize again the significance of the *relative* chelate stability defined by K^{M}_{MZ}/K^{Ca}_{CaZ}, we may briefly mention only 1,2-diaminocyclohexanetetraacetic acid (CDTA), which shows substantially higher stability constants than EDTA for most metal ions, but only slightly different relative stabilities. Its biological effectiveness, which has so far been tested for plutonium (3, 73), radiocerium (10) and radioyttrium (13), is thus in all respects comparable to that of EDTA and not superior to it. On the other hand, a particular group of polyamino acids were likely to be more effective than EDTA, and, as will be shown later, this hypothesis was confirmed experimentally. These are substances with an alkylene bridge longer than that of EDTA and interrupted by one or more atoms other than carbon.



X = O: 2:2′-bis[di(carboxymethyl)amino]diethyl ether (BADE, BAETA)

= S: 2:2′-bis[di(carboxymethyl)amino]diethyl sulphide (BADS)

= N: diethylenetriaminepentaacetic acid (DTPA)



X = O: 1:2-bis[di(carboxymethyl)aminoethoxy]ethane (BAE)

= S: 1:2-bis[di(carboxymethyl)aminoethylthio]ethane (BASE)

= N: triethylenetetraaminehexaacetic acid (TTHA)

The reduction of chelate stability due to lengthening of the alkylene bridge and enlargement of the chelate ring is offset by the hetero-atoms (ether oxygen, sulfur or substituted nitrogen) usable by the metal as ligand; most of the metals so far examined have shown K^{M}_{MZ}/K^{Ca}_{CaZ} values several log-units greater then EDTA (1, 5, 16, 21, 41).

The first substance of this group examined was BADE, which showed a stronger mobilization effect than EDTA for radiocerium, also in regard to its deposition in the skeleton (9, 10). This also proved to be the case with plutonium (33), but not with radioyttrium (13) or thorium (34). A substance with a considerably greater effectiveness and, in particular, a broader activity spectrum than the polyamino acids containing ether oxygen is diethylenetriaminepentaacetic acid (DTPA). Table 1 shows some representative results obtained from experiments with rats, which show clearly the considerable differences in effectiveness between EDTA and DTPA, even under conditions of delayed treatment. It should be added that the favorable results obtained with plutonium have been fully confirmed by other workers (25, 76), and that a greater effectiveness of DTPA as compared with EDTA is to be seen in the effect on iron (23, 24), chromium, cobalt, zinc (24), manganese (35) and uranium (8) toxicity.

The dependence of chelating agent effectiveness on the quantity E should present a straight line in the log-log plot, provided a certain threshold value for E has been exceeded, that is, the affinity of the radiometal for endogenous chelating agents is less than its affinity for a chelating agent used therapeutically. From the

TABLE 1. *Effect of $CaNa_3$-DTPA and $CaNa_2$-EDTA on Retention of Different Radioactive Metals in Rats*

Radionuclide	Chelate	Time of Injection After Radionuclide	% of Administered Dose			Ref.
			Liver	Skeleton	Excret.	
Y^{91}	control	—	3.4	56		(13)
	EDTA	2 minutes	1.7	25		
	DTPA	2 minutes	0.6	18		
	control	—	1.1	59		
	EDTA	7th, 9th, 11th, 13th day	0.7	48		
	DTPA	7th, 9th, 11th, 13th day	0.4	38		
Ce^{144}	control	—	42	30		(10)
	EDTA	2 minutes	13	32		
	DTPA	2 minutes	0.4	2.3		
	control	—	36	25		(11)
	EDTA	5th, 7th, 9th day	32	26		
	DTPA	5th, 7th, 9th day	2	16		
Th^{234}	control	—		60	1.5†	(34)
	EDTA	from 2nd to 5th day		60	5.1	
	DTPA	from 2nd to 5th day		43	26	
Pu^{239}	control	—	9.1*	6.6*		(75)
	EDTA	1 hour	4.9	4.6		
	DTPA	1 hour	2.7	0.9		
	control	—	7.9	64	2.4†	(33)
	EDTA	6th, 12th, 21st, 22nd, 25th, 26th day	11	46	6.7	
	DTPA	6th, 12th, 21st, 22nd, 25th, 26th day	1.6	40	20	
Am^{241}	control	—		40	40	(76)
	DTPA	6 hours		10	90	
	DTPA	7th day			80	

* Per cent/gm of dry tissue. † Urine.

definition of E it follows that this straight line represents the dependence of effectiveness both on the dosage of *one* particular chelating agent and on the relative chelate stabilities of a number of chelating agents. Thus the effectivities of two chelating agents having similar K values should overlap and, if adequate dosages are chosen, should behave identically. Figure 2 summarizes the corresponding data for radiocerium; four different polyamino acids (EDTA, DTPA, CDTA and HEDTA) were examined, six different dosages for DTPA being used. As regards skeletal effectiveness, it is possible to represent all experimental points within the limits of experimental error satisfactorily as a straight line, provided that E is greater than about 10^5. It should be stressed that with lower values for E the chelating agents induce a slight but statistically significant increase in radiometal deposition. The reason for this might be that the radiometal chelates are able to diffuse into the extracapillary space and thus bring enhanced amounts of radiometal into contact with the competing ion-exchanging or -adsorbing structures of the bone. Assuming, moreover, that the competition of the bone tissue is more important than that of the high molecular chelating

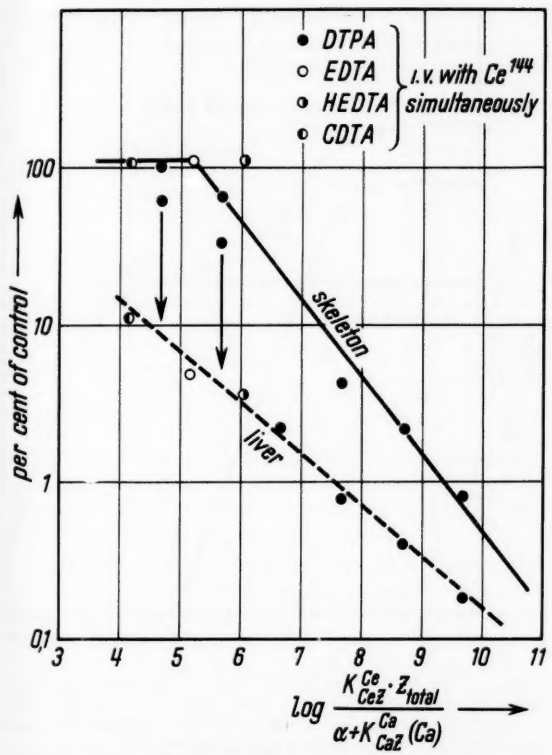


FIG. 2. Effect of several chelating agents and respective various doses of DTPA on the retention of Ce^{144} in the organs of the rat. Abbreviations: DTPA-diethylenetriaminepentaacetic acid, EDTA-ethylenediaminetetraacetic acid, HEDTA-hydroxyethylethylenediaminetriacetic acid, CDTA-1,2-diaminocyclohexanetetraacetic acid (Catsch and Lê (10), Catsch, unpublished results).

agents of the blood plasma, there must be a tendency towards increased deposition in the skeleton. As to effectiveness in the liver, the two lowest DTPA dosages of 10^{-8} and 10^{-9} mole/animal show a definite deviation from linear regression. The fact that extremely low dosages of chelating agent are less effective than expected is not surprising in view of the fact that an important prerequisite for the validity of the dependence of effectiveness on E, namely a considerable excess of chelating agent over the concentration of radiometal and of biological trace metals, is lacking in this case. It might be thought that appropriate extrapolation of these curves would throw light on the E values necessary to obtain still greater mobilization. However, a certain caution seems indicated here in view of the fact that the postulated dependence of effectiveness on E leaves a number of relevant factors out of account and so implies a certain degree of over-simplification. Our starting point hitherto has been that the behavior of the chelating agents within the organism is completely inert, that is, they are neither decomposed nor deposited but are within a short time excreted completely and unchanged. This is indeed true for polyamino acids like EDTA and DTPA, as Foreman (24, 30, 32) has shown, but does not apply necessarily or unconditionally to other chelating agents of different chemical structures.

The extent to which the efficiency pattern depends on the metabolic behavior of the chelating agent is strikingly shown by the condensed phosphates, regardless of whether we think of these (like Graham's salt) as chelating agents or, with Thilo (79), as soluble ion exchangers. As can be seen from Table 2, the polyphosphates when administered early have a fairly strong effect on the behavior of various radiometals, deposition in the bone tissue being reduced as compared with EDTA, while the effectiveness in the parenchymatous organs is considerably less or indeed deposition may be actually greater (10, 13, 73). Tests with polyphosphates labeled with P^{32} have shown (38) that they are present in the blood as nondiffusible and nonultrafilterable colloids and are deposited in the organs of the reticuloendothelial system and the kidneys. This also explains the increased deposition of radiometals in these organs and the unexpectedly high skeletal effectiveness. The fact that the condensed phosphates do increase the excretion of radiometals from the organism is explained by the hydrolytic breakdown of the polyphosphates to lower molecular poly- and metaphosphates, which can be excreted and have complexing properties (10, 73). Since the condensed phosphates have only a narrow therapeutic range and their effect in delayed treatment is negligible (10), they are of little practical importance.

An unexpected result was also found when the higher homologue of DTPA, ten-dentate triethylenetetraminehexaacetic acid (TTHA) was examined, (Table 3). TTHA if administered early inhibits deposition of radiocerium and radioyttrium in the skeleton much more than DTPA. On extrapolation of the appropriate curve in Figure 2 this would correspond to an E value of the order of 10^{10} or 10^{11}. As regards effectiveness in the liver, a reduction of the radiocerium content to less than 0.1 per cent of the control would be expected, whereas, what we have in fact is a reduction merely to 0.25 per cent. As TTHA has a number of ligand sites greater than the coordination number of the radiometals concerned, we must assume the formation to some extent of bimetallic chelates and, in certain circumstances, of polynuclear aggregates, which could lead to

TABLE 2. *Effect of 25 mg Sodium Polyphosphate (Graham's Salt) on Retention of Different Radioactive Metals in Rats**

Radionuclide	% of Control, Fiducial Limits ($P = 0.05$)			Ref.
	Liver	Kidneys	Skeleton	
Y^{91}	132 (103–168)	450 (198–980)	51 (44–59)	(13)
Ce^{144}	23 (20–26)	250 (182–345)	48 (41–55)	(10)
Pu^{239}	82 (57–107)	189 (122–256)	34 (25–43)	(73)

* Injection ip 2 minutes after Y^{91} and Ce^{144}, 10 minutes after Pu^{239}.

TABLE 3. *Effect of DTPA and TTHA on Distribution of Ce^{144} and Y^{91} in the Organs of the Rat**

Radionuclide	Treatment	% of Control, Fiducial Limits ($P = 0.05$)	
		Liver	Skeleton
Ce^{144}	DTPA after 2 minutes ip	1.02 (0.78–1.30)	10.1 (8.8–11.6)
	TTHA after 2 minutes ip	1.50 (1.31–1.73)	5.20 (4.45–6.12)
	DTPA simultaneously iv	0.11 (0.08–0.14)	1.28 (0.92–1.79)
	TTHA simultaneously iv	0.23 (0.17–0.31)	0.45 (0.33–0.62)
Y^{91}	DTPA after 2 minutes ip	36 (27–49)	5.70 (5.00–6.50)
	TTHA after 2 minutes ip	42 (31–60)	3.27 (3.21–4.37)

* Dosages: 250 μM $CaNa_3$-DTPA ip; 100 μM $CaNa_3$-DTPA iv; 250 μM $CaNa_4$-TTHA ip; 100 μM Ca_2Na_4-TTHA iv (Catsch & Schindewolf-Jordan, unpublished results).

behavior similar to that of the colloidal condensed phosphates. A deviation in the behavior of TTHA was also established in the course of our investigation (8) into the influence of various chelating agents on the acute toxicity of uranyl nitrate: when a single dose of the chelating agent is administered early, the LD_{50} of uranium is increased from 6.7 mg/kg to 12.4 mg/kg by EDTA and to 16.2 mg/kg by DTPA. On the other hand TTHA, the toxicity of which is normally no different from that of EDTA or DTPA, was 100 per cent lethal to uranium injected animals, and, actually, within one hour of administration. We attributed this potentiation of TTHA toxicity by uranium to the formation of insoluble polynuclear uranium chelates in the blood. With regard to the figures given in Table 3, which show a clear increase of effectiveness as against DTPA, we feel that this substance calls for further investigations, which should also include plutonium.

To deal with the question of the dependence of effectiveness on the quantity of E, any deviations from linear behavior can be explained on different principles from those discussed earlier. There would seem to be some grounds for assuming that a given organ does not behave as a homogeneous unit in the absorption of radiometals, but rather as one composed of several compartments differing in affinity for radiometals and so, also, in the ability to mobilize the radiometal by means of chelating agents. Figure 3 shows schematically how the assumption that the organ consists of two compartments with different affinities for a radiometal and thus, also, different threshold dosages for E results in a nonlinear relation between effectiveness and E for the organ as a whole.

Let us now turn to radiostrontium as a special case. Here DTPA proved just as ineffective as EDTA (14, 51); comparable experiments on radiobarium and radium have still to be made, but it can be presumed with fair probability that DTPA will also fail with these. The

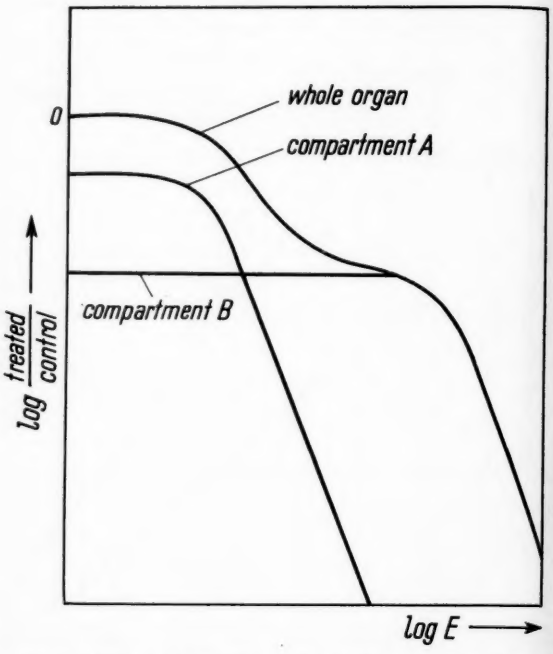


FIG. 3. Hypothetical dependence of the effectiveness of a chelating agent on the value E (see p. 207) under the assumption that the organ is subdivided in two distinct compartments characterized by a different affinity towards the radiometal.

total failure, or at best only slight effectiveness, of all chelating agents so far tested with radiostrontium is not fortuitous (as Schubert (68) has recently shown again in detail). Rather it is an expression of the general rule that chelate stability constants are inversely proportional to the ion radius of the alkaline earth atoms, in other words, that the competition of the Ca ions for the chelating agent will be notably stronger than in the case of other radiometals. It must be granted that the difference between the Ca and Sr stability constants of EDTA and DTPA, of some 2 log units, is exceptionally large, and that it is not difficult to find chelating agents with E values only a little below 1. But the effectiveness of these is still disappointingly low, the reduction of radiostrontium deposition in the skeleton being 10–20 per cent on early administration. Individual chelating agents which have been shown to have some, if only slight, effectiveness are sodium citrate (7, 14, 45–47, 70), tricarballylic acid (45–47), sodium thiosulfate (15), pyrocatecholdisulfonic acid (14, 51), low- and high-molecular meta- and polyphosphates (7, 14), BADS (14) and BADE (14, 53). Since rhodizonic acid is a selective precipitating agent for strontium, Lindenbaum and Fried (55) examined its effect on the distribution of carrier-free radiostrontium but found it relatively slight. This, moreover, was not confirmed by other investigators, admittedly working under somewhat different experimental conditions (51, 57, 81). So far the greatest reduction in radiostrontium deposition in the skeleton

TABLE 4. *Effect of BADE (BAETA) and BADS on Skeletal Retention of Sr^{85} in Rats (Catsch, unpublished results)*

Treatment	% of Control Fiducial Limits ($P = 0.05$)
250 μM Na_2-BADS simultaneously	82 (77–86)
250 μM $SrNa_2$-BADS simultaneously	66 (63–70)
500 μM $SrNa_2$-BADS simultaneously	61 (56–67)
100 μM Na_2-BADE simultaneously	89 (80–99)
100 μM $SrNa_2$-BADE simultaneously	71 (64–79)
30 μM $SrNa_2$-BADE simultaneously	86 (82–91)
250 μM $SrNa_2$-BADE simultaneously	66 (62–71)
500 μM $SrNa_2$-BADE simultaneously	52 (49–55)
500 μM $SrNa_2$-BADE after 2 hours	84 (76–92)
500 μM $SrNa_2$-BADE after 24 hours	93 (85–102)

(to 35 per cent of the control) has been obtained with Diamond Fast Blue (51); but since this dyestuff in effective doses has proved strongly toxic and sometimes lethal it has not any practical importance.

We cannot confirm Schubert's assumption (70) that the removal of radiostrontium by means of zirconium citrate is governed exclusively by the citrate component since we have shown (14) that zirconium citrate is definitely more effective (with a reduction to 65 per cent) than sodium citrate (80 per cent). According to Kroll (53) BADE shows a greater effectivity when used in the form of the Sr chelate, that is to say, the effect of the chelating agent is combined with that of an isotopic dilution of the radiostrontium. Table 4 shows that we were able to confirm that the effectiveness of Sr-BADE was greater than that of Na_2-BADE, though still inadequate, especially if it was administered several hours after radiostrontium. Naturally a low mobilization effect is better than none at all; but the results obtained so far are so insignificant (compared with those obtained by DTPA with other radiometals) that in my estimation there is at present no occasion to revise the generally pessimistic assessment of the therapeutic possibilities for radiostrontium poisoning; especially as the chances of finding a chelating agent with considerably higher relative stability or, indeed, with a selective affinity for strontium are extremely slight. There is the additional fact that the effective substances mentioned above prove ineffective if administered after a delay. Their ineffectiveness in delayed treatment is attributable mainly to the fact that the radiostrontium which is initially reversibly bound on the surface of the apatite crystals is relatively soon removed from the equilibrium as a result of the onset of recrystallization and is converted into a form which is no longer accessible to the chelating agent (60).

Since heavy metals of the sixth period prefer sulfur to oxygen as ligand atom, it seemed useful to include suitable substances in our tests. In conformity with the only slightly differing $K^{Pb}_{PbZ}/K^{Ca}_{CaZ}$ values of EDTA and DTPA both these chelating agents showed no significant variations of effectiveness either on early or delayed administration; Fried *et al.* (37) obtained the same result in exploratory toxicity experiments. Polyamino acids, which contain ether or thioether linkages, also showed no increase of effectiveness over EDTA. It should of course be mentioned here that sulfur in the form of thioether is in principle a much weaker coordination partner than in the form of mercaptide or sulfite. The only polyamino acid which gave us a definitely stronger mobilization of radiolead than EDTA was cystaminetetraacetic acid (CyTA). It is not yet clear whether CyTA is effective in itself or only after splitting of the disulfide bond, that is, in the form of the resulting mercaptoethyliminodiacetic acid. This latter assumption would seem to be supported by the toxicity of CyTA, which is about ten times higher than that of other polyamino acids and appears, primarily, in damage to the kidneys. The stability constants for the Pb-CyTA chelate have not yet been established, though that for mercaptoethyliminodiacetic acid is known (4). Assuming a complete splitting of the CyTA in the organism, and considering the effectiveness in relation to the E values for various dosages of mercaptoethyliminodiacetic acid, EDTA and DTPA, we get (as shown in Fig. 4) a satisfactory linearity within the limits of experimental error in the case of the liver and kidneys. As to the skeleton, CyTA proves considerably less effective than its behavior in the other organs might have led us to expect, the slope of the straight line obtained for various dosages of CyTA being apparently identical with the slope representing the effectiveness of various dosages of EDTA and DTPA. To explain this discrepancy we may assume as a working hypothesis that the splitting of the disulfide bond of CyTA occurs preferentially in the liver and kidneys, or that the mercaptoethyliminodiacetic acid has a specially high affinity for these organs. Results so far do not suggest that CyTA is of practical importance since its effectiveness in nontoxic dosages is of the same order as that of EDTA and DTPA.

The first results of experiments still in progress with carrier-free mercury-203 are shown in Figure 5. The only organs whose mercury content is affected by polyamino acids, and that only to a slight extent, are the kidneys. The graph connecting effectivity and the E values shows, in this case, only a negligible slope and a marked scattering of the points. One has the impression that it is the longer-chain polyamino acids that are considerably less effective than would have been expected from their E values. The reasons for this exceptional behavior are not yet clear; it is possible that the substances in question behave differently from the other polyamino acids in metabolism. The polyamines which we also examined were completely ineffective in spite of very much higher E values, which is most probably due to their metabolic breakdown. As with radiolead, CyTA proved the most effective, the mercury content of the kidneys being reduced, under identical test conditions, to about 25 per cent of the control, although a much slighter effect might have been expected from the E value of 10^8 (on the assumption that the mercapto-

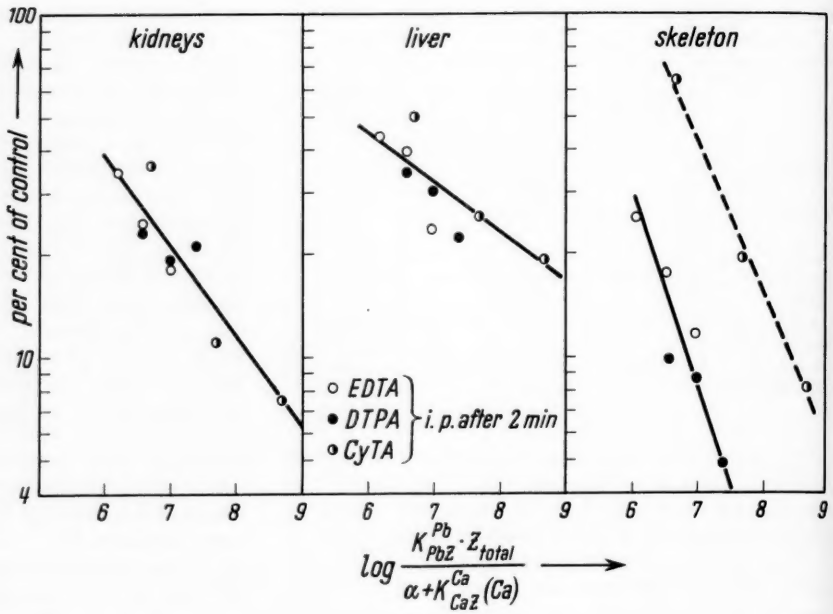


FIG. 4. Effect of various doses of several chelating agents on the retention of Pb^{210} in the organs of the rat. It is assumed that CyTA (cystaminetetraacetic acid) is completely split up into 2-mercaptoethyliminodiacetic acid (Catsch, unpublished results).

ethyliminodiacetic acid is the effective substance). It is interesting that other chelating agents with fewer ligand sites, such as iminodiacetic acid and ethylenediamine-N,N′-diacetic acid were effective to a relatively high degree. As Figure 6 shows, the effectiveness of CyTA when administered after a delay increased, in contrast to what happened with other chelating agents and other radiometals. It was stated at the outset that both toxicity and excretion of polonium-210 are favorably affected by BAL (43, 44) or its water-soluble sulfonic acid derivatives (22). No comparison has yet been made between the sulfhydryl compounds and DTPA or other polyamino acids.

The high effectiveness of DTPA, which is evident even at relatively low dosages, makes it possible to analyze its action in more detail than is possible with EDTA. Table 1 shows that the greater effectiveness of DTPA (as against EDTA) is maintained when it is administered after delay, but that the dependence of effectiveness on time is again unmistakable. To explain this generally valid circumstance a factor which has been ignored hitherto must now be taken into account, namely the rate at which the chelates are excreted from the organism. The high effectiveness observed on administration of the chelating agent immediately or within a short time is partly due to the fact that the freely diffusible metal chelate is excreted rapidly by the kidneys and thus is to a large extent withdrawn also from the competition of the endogenous chelating agents and ion exchangers. If a chelating agent is removed comparatively slowly from the blood, the partition of the metal chelate between excretion and intercellular space is shifted in favor of the latter, and in the light of the ideas discussed above we should expect a reduction of effectiveness. That this assumption is correct has been

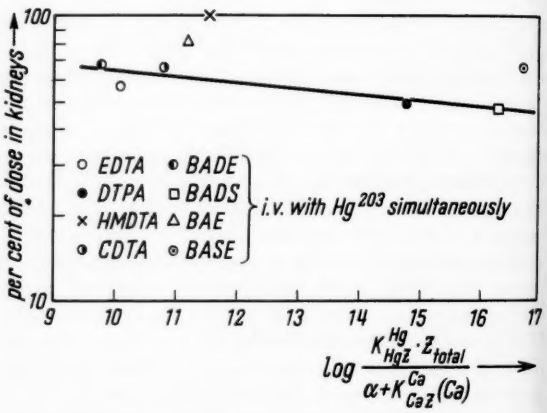


FIG. 5. Effect of several chelating agents on the retention of Hg^{203} in the kidneys of the rat. HMDTA-hexamethylenediaminetetraacetic acid; for further abbreviations see p. 208 and legend of Fig. 2 (Catsch and Nigrović, unpublished results).

impressively shown in a test carried out by Semenov *et al.* (72): Carrier-free radioyttrium was injected with an excess of EDTA into nephrectomized rats and the radioyttrium content of the organs was measured after 24 hours. The distribution differs hardly at all from that in the control animals which were given only radioyttrium. It should be mentioned that the blood concentration of the EDTA labeled with C^{14} remains constant over the same period of time in the case of the nephrectomized animals (32). This shows that as soon as the excretion factor is removed the stability of the Y-EDTA chelate is no longer sufficient to compete successfully with the tissues.

If a chelating agent is administered when the bulk of

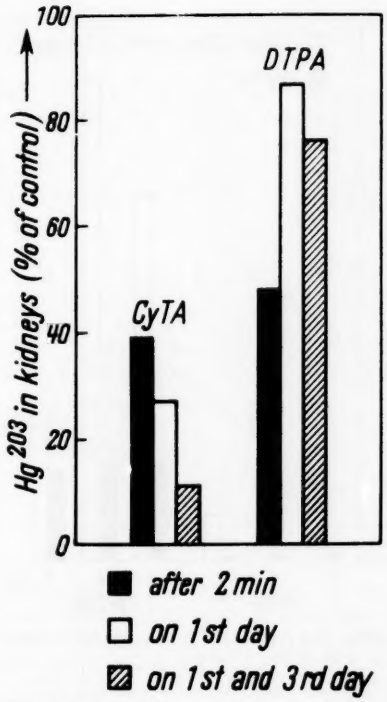


FIG. 6. Effectiveness of DTPA and CyTA (50 μM per rat and dose) in removing Hg^{203} from the kidneys of the rat (Catsch and Nigrović, unpublished results).

the radiometal is already deposited in the tissues and organs, its effectiveness depends in the first place on the relationship defined above between the various complexing affinities and also on the chelate excretion rate, which, however, here has the opposite effect to that discussed above. Mobilization of the radiometal deposited in the tissues is in principle possible only through chelation of the free metal ions and the consequent disturbance of the equlibrium with the cellular chelating agents or ion exchangers. If the rates at which the endogenous chelates decompose and/or the metal ions flow into the extracellular space are slow in relation to the rate of excretion of the chelating agent, then the effectiveness of the latter must decrease fairly rapidly. Provided a chelating agent is effective at all, that is, it exceeds the threshold value for E for the radiometal concerned, its effectiveness should be inversely proportional to the excretion of the chelate. The significance of the solubilizing rate may be exemplified by the findings of Schubert and Fried (69): the dissolution of an olated thorium hydroxide by means of DTPA *in vitro* is extremely slow in spite of the very high stability of the Th-DTPA chelate. The same thing occurs *in vivo*; if animals are injected with colloidal thorium hydroxide, excretion is only slightly intensified by repeated doses of DTPA, whereas if monomeric thorium citrate is injected the effectiveness of the DTPA becomes very marked indeed. The reduced effectiveness of chelating agents with certain isotopically diluted radiometals [e.g. radioyttrium (72)] is also attributable to the slow solubilization of the colloidal metal aggregates.

The excretion of radiometals from the various organs as a rule represents a multiexponential function in which the individual terms formally can be assigned to particular compartments which presumably possess different affinities for combination with the radiometal. Here it is of course a prerequisite that individual compartments are not interchangeable or that the appropriate rates of interchange are low in relation to the excretion rate. Since the mobilizing capacity of a chelating agent depends essentially on the stability of the endogenous radiometal complexes, it follows that the effectiveness is inversely proportional to the so-called biological half-time, and should decrease in the event of a multiexponential excretion function, indicating the progressive increase in the significance of the "slow" components with the passage of time. These suppositions were confirmed by tests with radiocerium, whose excretion from the liver during the first two months after administration can be expressed as the sum of two exponential terms with half-time values of 5 and 15 days, respectively, in rats. The "fast" component is predominant, that is, during the first four weeks, the effectiveness of single dosages of DTPA remains roughly constant, giving a fall in radiocerium content to about ⅓, regardless of the time of administration (Table 5). It decreases only when the "slow" component is predominant. This is also the case when the readily mobilizable fraction of the radiocerium, corresponding to the "fast" component, has been removed by a previous dosage of DTPA, so that the effects of several successive DTPA dosages are no longer fully additive (Catsch, unpublished results). For the reasons already discussed in connection with radiostrontium, the progressive decrease in effectiveness in the skeleton is especially strongly marked. Examination of the distribution over the various sections of the femur has shown that radiocerium deposited in the metaphysis is mobilized by DTPA to a greater extent than from the epiphysis and diaphysis (Table 6). It can also be seen that here the progressive loss of effectiveness is more strongly marked, as would be expected given the exceptionally intensive growth processes taking place in the metaphysis. These results were obtained with young rats, and it may be asked whether the dependence of effectiveness on time would be as great if the animals were fully grown. Another factor which is still not clear is the effectiveness of chelating agents where the administration of radiometals has been maintained over a prolonged period, the result of which, as compared with a single dosage, is to give a much more uniform distribution of the radiometals in the skeleton.

The differences in the extent to which the radiometal in the liver and skeleton can be depleted by DTPA were shown by Fried *et al.* [quoted in (65)] for plutonium also: daily administration of DTPA over two weeks reduced the plutonium content of the liver to 3 per cent of the control, while that of the skeleton fell only to 50 per

TABLE 5. *Dependence of the $CaNa_3$-DTPA Effectiveness in Removing Ce^{144} from the Organs of Rats on the Time of its Administration**

Day of Administration	% of Control, Fiducial Limits (P = 0.05)		
	Liver	Kidneys	Skeleton
1st	21 (19–24)	54 (43–68)	72 (64–80)
3th	28 (23–33)	86 (63–117)	72 (62–82)
25th	24 (14–39)	84 (64–111)	90 (83–98)
7th, 9th, 11th, 13th	10 (7.6–13)	55 (43–68)	59 (52–68)
41st, 43rd, 45th, 47th	28 (18–43)	98 (80–123)	101 (90–112)

* Single ip dose: 250 μM (Catsch, unpublished results, and Catsch & Lê (11)).

TABLE 6. *Effect of $CaNa_3$-DTPA on Retention of Ce^{144} in Different Parts of the Rat Femur**

Time of Injection After Ce^{144}	% of Control, Fiducial Limits (P = 0.05)		
	Epiphysis	Metaphysis	Midshaft
2 minutes	14 (11–17)	9 (7–11)	12 (10–14)
5th day	87 (70–108)	64 (50–82)	82 (70–97)
5th, 7th, 9th days	71 (58–86)	46 (40–53)	72 (55–87)
25th day	90 (80–100)	85 (63–115)	90 (70–115)

* Single ip dose: 250 μM (Catsch & Schindewolf-Jordan, unpublished results).

cent. Tests by Foreman (25), in which administration of DTPA was begun one month after administration of plutonium and was continued over various periods of time, also suggest that the effectiveness of DTPA was pretty well exhausted after a four-week treatment. As to its effectiveness in man, we have so far only the observations of Norwood; in some cases, where the incorporation of plutonium took place several years previously, DTPA gave a considerably more (about 10 times) intensified excretion as against EDTA.

Absorption of DTPA from the gut is low (as with EDTA) and represents about 4 per cent of the total dosage (24). Provided the dosage is sufficient the fraction absorbed should be enough to show a clear effect. The effectiveness of DTPA administered orally has been experimentally shown for radiocerium (12) and in a particularly impressive manner by Foreman [quoted in (65)] for americium-241.

In contrast to the previously accepted view that the excretion of radiometals mobilized by polyamino acids is effected only by the kidneys, it has been shown that DTPA causes a marked increase in radiometal excretion in the feces, especially in the case of those which, like radiocerium, are deposited to a large extent in the liver (Fig. 7). After a single dosage of DTPA, for example, on the fourth day after administration some 11 per cent of radiocerium (above the control excretion rate) was excreted in the urine and 20 per cent in the feces. Because the fecal excretion rate of radiocerium is naturally high, the factor by which the pretreatment excretion level is increased by DTPA is considerably higher in urine. The

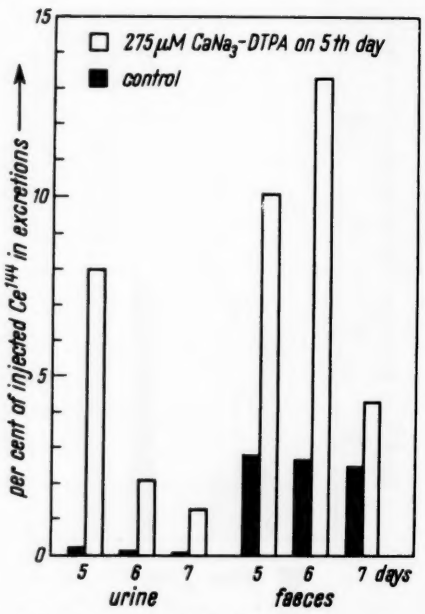


FIG. 7. Effect of a single DTPA dose on the excretion of Ce^{144} from the rat (Catsch and Lê (11)).

proportion of radiocerium mobilized by DTPA and excreted in the urine and feces is about 1:2, which is surprising in view of the fact that the corresponding proportion of excreted C^{14}-labeled DTPA is about 16:1 (24).

DTPA gave unexpected results in other respects as well. Earlier work (18, 72) had shown that EDTA is still effective when administered three hours *before* the administration of radioyttrium. In regard to the clear effectiveness of relatively small dosages of DTPA (cf. Fig. 2) a prophylactic effectiveness might be expected at even longer intervals of time and has in fact been established experimentally (12): 250 μM DTPA per rat, injected 12 hours before administration of radiocerium, reduced deposition of the latter in the skeleton to about 50 per cent and in the liver to 3 per cent of the control values. In this case the very pronounced liver effectiveness is surprising. According to Foreman (24), after 12 hours some 0.03–0.05 per cent of the total Ca-DTPA dosage is still present in the blood. This would in our experiment be equivalent to a dosage of about 0.1 μM, from which, however, on the basis of the results discussed above (cf. Fig. 2), one would have expected the liver content to fall to not less then 10–20 per cent of the control value.

From the earlier work on EDTA it was assumed that the enhanced excretion, in keeping with the rapid and complete excretion of EDTA, was limited to a fairly short period, about the first 24 hours. Fried *et al.* (33) however showed that plutonium clearly continued to be excreted in the urine in more than normal quantities several days after administration of DTPA. Our own tests with radiocerium had given the same results (Fig.

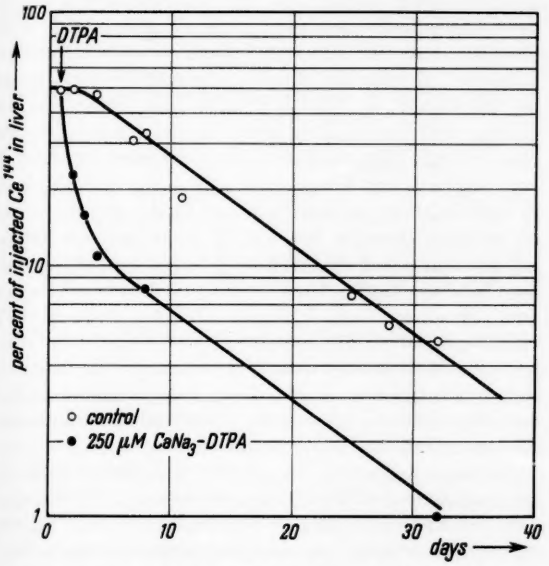


FIG. 8. Effect of a single DTPA dose given on the 1st day on the Ce^{144} content in the liver of the rat (Catsch, unpublished results).

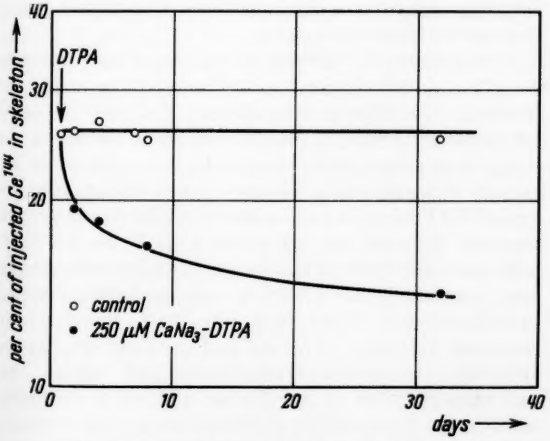


FIG. 9. Effect of a single DTPA dose given on the 1st day on the Ce^{144} content in the skeleton of the rat (Catsch, unpublished results).

8, 9): after a single dose of DTPA on the first day the liver shows a progressive decline in radiocerium content which continues over at least five days, and only on the eighth day does the effectiveness of DTPA appear to be exhausted, as thenceforward the curve runs parallel to the excretion curve of the untreated controls. In the skeleton the decrease is definite even after the eighth day. Here, of course, it is questionable whether this is due solely to the DTPA, and we suggest the following explanation: we know that a certain fraction of the radiocerium initially deposited in the liver returns to the blood stream and is absorbed into the skeleton. The constancy of the radiocerium content in the skeleton is thus only apparent, because the mobilization of radiocerium from the bones is to a large extent compensated by this redistribution, and cannot be observed until this factor has been removed, that is, after the radiocerium content of the liver has been reduced by DTPA.

The somewhat unexpected results discussed above—the partition of radiometal excretion between urine and feces, the surprisingly prolonged or high effectiveness after a single or prophylactic dosage of DTPA—seem to call for some revision of the accepted views on the behavior of polyamino acids in the organism. Since the Ca chelates of polyamino acids are water-soluble and usually anionic, it seemed from the first improbable that they would be able to penetrate cell membranes and enter the intracellular space to any great extent. This assumption was supported by the work of Foreman (24) on the behavior of Ca chelates labeled with C^{14}. Our own observations on the other hand could well be explained by the assumption that a fraction of the Ca-DTPA chelate, minor indeed but still effective, tends to concentrate in the intracellular space, at any rate, of the liver.

This tentative hypothesis is also favored by other observations that are not in a strict sense proof positive and only indirect: in the first place, the DTPA concentration in the liver 24 hours after administration is clearly higher than that in the blood (24). Studies by Lindenbaum and Schubert (56) on tissues from animals given plutonium show that the ultrafilterability of plutonium present in the tissues is increased following a single DTPA dosage and that this persists for at least a week. The information given does not of course show how the organ homogenates examined were obtained and prepared, so that the increased diffusibility of the plutonium could naturally be due also to traces of DTPA in the extracellular space. Our own work on radiocerium, in which the liver was thoroughly and very carefully perfused *in situ* before being homogenized, gave the same result: 5–12 per cent of the total amount of radiocerium contained in the liver homogenate remained freely dialyzable during the first five days after administration of the DTPA, whereas in the untreated control animals this fraction was practically nil.

We may also mention the results of work still in progress, which deals with the effect of DTPA on the intracellular distribution pattern of radiocerium. A state of equilibrium, that is a constant distribution of radiocerium over the various subcellular fractions of the liver homogenate, is reached only some days after the injection of radiocerium; during the first few days the protein-free fraction of the cytoplasm shows a fairly high and slowly decreasing radiocerium concentration, while that of the cytoplasm proteins and of the cellular organelles increases not only relatively but absolutely over the same period. It can thus be assumed that the supernatant obtained after precipitation of the cytoplasm proteins represents the quickly reacting cell fraction, and that the transfer of the radiocerium into the other subcellular fractions and/or its complexing by the

appropriate constituents take place relatively slowly. On the assumption that the Ca-DTPA chelate is present only in the extracellular space, it would be natural to expect the administration of DTPA to be followed by a reduction of radiocerium content of the protein-free cytoplasm fraction, at the very least equivalent to that in the slower reacting fractions. However, Figure 10 shows that in fact the opposite is the case: the radiocerium concentration of the cytoplasmic supernatant is clearly relatively higher during the first few days after administration of DTPA as compared with the other fractions, and shortly after administration is absolutely higher. This observation, together with the evidence obtained by electrophoresis for the presence of Ce^{144}-DTPA in the cytoplasm, favors our assumption that a small fraction of the DTPA is present within the cell for a fairly long time. At present we are trying with the help of autoradiographic investigations to show direct evidence of the presence of DTPA labeled with C^{14} in the cell. It seemed to me necessary to discuss more closely these observations, which at first sight seemed of only theoretical interest, because (as will be shown later) it afforded starting points of practical importance for further development.

The polyamino acids are as a rule administered in the form of their Ca chelates, which are only slightly toxic. As the free sodium salts give rise to some demineralization of the skeleton and as sufficient clinical experience is available to show the tolerance for Na_2-EDTA when slowly administered intravenously, the question arises whether use can be made of this to improve the effectiveness of the chelate. Cohn *et al.* (18) thought that the relatively effective mobilization of radioyttrium from the skeleton observed by them could be attributed to the alternating administration of $CaNa_2$- and Na_2-EDTA; there are no controls in this case, so that this conclusion does not seem justified as yet. Semenov and Tregubenko (72) have recently dealt in detail with the same question and were able by means of repeated doses of Na_2-EDTA to reduce the radioyttrium content of the skeleton to 64 per cent of the control, whereas $CaNa_2$-EDTA reduced it only to 80 per cent. However, both forms of EDTA proved equally effective as far as the parenchymatous organs were concerned, which is what would be expected. This observation is not however amenable to generalization and it was impossible to reproduce it with other radiometals such as cerium and plutonium.

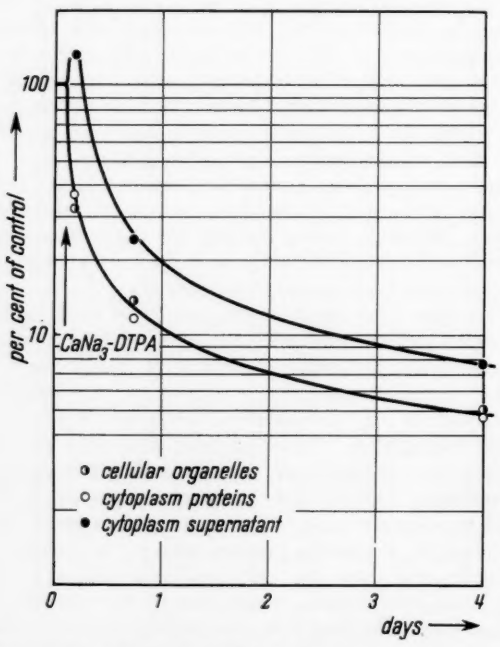
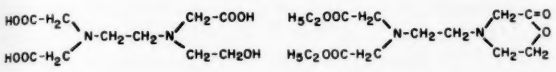


FIG. 10. Effect of a single DTPA dose given after 2.5 hours on the Ce^{144} content in the subcellular fractions of the liver homogenate (Catsch and Immel-Teller, unpublished results).

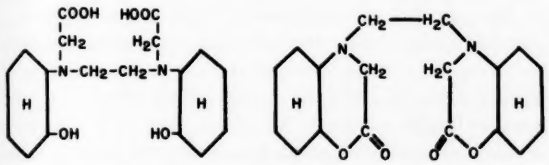
Attempts to increase the effectivity of chelating agents by simultaneous administration of diuretics, the cholagogue dehydrocholic acid or parathormone (11, 14, 39, 72), which intensify the bone resorption processes, have proved negative or unconvincing. A clear increase in the effectiveness of EDTA, however, was obtained in the mobilization of lead from the bones by combining it with vitamin A (59), which in massive dosages is known to enhance bone resorption.

A fundamentally different starting point for improving the therapeutic effectiveness of chelating agents is to be found in the observations already discussed in detail concerning the distribution of chelating agents in the extra- and intra-cellular space. In our view there are certain indications which suggest that the high effectiveness of DTPA is to a certain extent due to its penetration into the intracellular space, particularly in the liver; and it was therefore natural to expect that if this process were intensified there would be a corresponding increase in effectiveness. This possibility had already been discussed by Fried *et al.* (36) and even verified experimentally: by administering fluoroacetic acid, these authors were able to reduce the toxicity of nonradioactive lead by a factor of about 1.15. The mode of action of fluoroacetic acid is supposed to be that, as a metabolic inhibitor, it interferes with the Krebs cycle and leads to an intracellular accumulation of citrate, which is known to have a fairly high affinity for lead ions. The stability of the citrate chelates with the radiometals of practical interest to us is however too low to suggest that this principle could be satisfactorily applied in this case.

Another approach that seemed more promising was to use chelating agents with a high intrinsic permeance, for example esterified polyamino acids. By esterifying the carboxyl groups, the ligand groups necessary for chelation were, it is true, blocked, but it was reasonable to expect that the esters would be hydrolyzed at the intracellular pH, perhaps with the assistance of esterases, and that the effective ligand groupings would be liberated.



N′-(2-hydroxyethyl)ethylenediamine-N,N,N′-triacetic acid (HEDTA) and derivative.



N,N′-bis (2-hydroxycyclohexyl)ethylenediamine-N,N′-diacetic acid (DOC).

Among compounds which we have so far examined is the ethyl ester of HEDTA, which exists partly in a lactone form, and thus is a so-called inner ester. The compound is water soluble at pH 7 and is readily administered intravenously. The corresponding free polyamino acid, HEDTA, is a chelating agent comparable with EDTA 9, 13) in biological effectiveness. Also water soluble at pH 7 is the second compound examined by us, DOC, which occurs as a dilactone. As a control DOC was administered in the form of its $CaNa_2$ chelate which was prepared by hydrolyzing the lactone at pH 10 and 50°C and then adding an equimolar amount of Ca. Table 7, which summarizes the exploratory tests, shows that both esters 24 hours after intravenous injection of radioyttrium or radiocerium effect a clear mobilization of the radiometals from the liver, while the corresponding Ca chelates have no significant effect. An approximately equivalent effect was observed when the ethyl ester of HEDTA was administered orally. That the ester has no effectiveness in the skeleton is consistent with the basic suppositions about its mode of action. That no clear effect was established in the kidneys does not quite justify the conclusion that the esters are selectively taken up by the liver cells and/or are hydrolyzed by them. The results mentioned earlier had shown that radiocerium deposited in the kidneys is fairly quickly converted into a form that is difficult to mobilize (see Table 5). In the light of the observation that fecal excretion is intensified by the DTPA—present, as assumed, in the intracellular space—this was also to be expected in the case of esterified polyamino acids and was established experimentally (Fig. 11). The reduction of the radiometal content of the liver by some 30 per cent by the esters is in these circumstances rather low and not comparable with the effectiveness of DTPA, which in all probability is connected with the inadequate relative stability of the HEDTA and DOC chelates. These observations are, therefore, at present of practical importance only to the extent that they indicate a fundamentally new approach to the therapeutic use of chelating agents. Final conclusions can naturally only be drawn when we know something about the esters of more powerful chelating agents, such as DTPA.

Hitherto we have been concerned solely with the removal of radiometals already deposited in the organs or—under optimum conditions—still in the blood stream at the time when the chelating agent was administered. This leaves out of account a point of practical importance, namely the way in which contamination occurred. The experience of the past few years (28, 29, 54, 64) has shown that the inhalation of radioactive material and contamination of wounds are in practice by far the commonest ways. If the radiometals entering the body in this way show only a slight absorption rate and if in the case of contaminated wounds the radioactive deposit cannot be removed by surgical methods, an attempt must be made to intensify absorption with simultaneous excretion of the radiometal in order to prevent excessive local radiation. There can be no doubt that even in this special case chelating agents are the most promising means. Appropriate experimental investigations into this problem (19, 31, 40, 63) have so far been limited

TABLE 7. *Effect of HEDTA and DOC, Administered after 24 Hours as Calcium Sodium Chelate (Ch) or Respective Ester Lactone (E), on Distribution of Ce^{144} and Y^{91} in the Organs of the Rat**

Radio-nuclide	Treatment	% of Control, Fiducial Limits (P = 0.05)		
		Liver	Kidneys	Skeleton
Ce^{144}	HEDTA-Ch iv	98 (93–103)	90 (84–97)	99 (93–105)
	HEDTA-E iv	76 (73–79)	94 (88–101)	102 (97–107)
	HEDTA-E po	79 (73–86)	112 (96–130)	106 (96–117)
	DOC-Ch ip	87 (75–100)	103 (72–147)	98 (84–115)
	DOC-E ip	68 (59–79)	91 (63–130)	93 (79–109)
Y^{91}	HEDTA-Ch iv	94 (80–111)	98 (77–129)	90 (85–96)
	HEDTA-E iv	67 (56–79)	114 (87–150)	96 (91–101)

* 100 μM HEDTA, 250 μM DOC (Catsch).

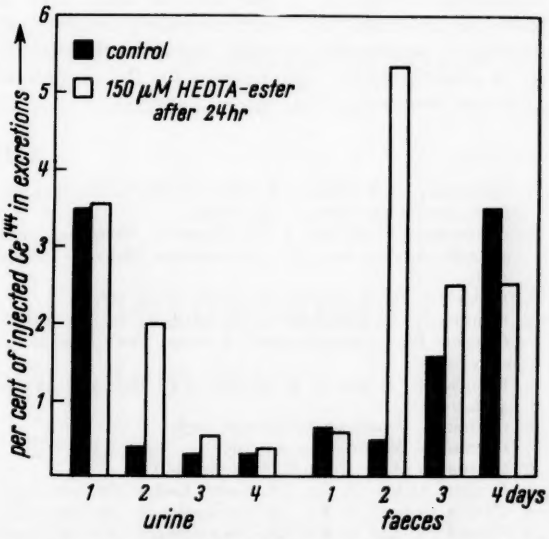


FIG. 11. Effect of a single HEDTA-ester dose on the excretion of Ce^{144} from the rat (Catsch, unpublished results).

and carried out only with EDTA. They show, as does also analogous experience with man (64), that it is indeed possible in principle to intensify absorption in this way, but that the decontamination which is the final objective remains disappointingly slight, so that, first of all, it is not easy to decide whether the inherently low effectiveness of EDTA is to be blamed exclusively for the poor results obtained. The examination of DTPA which is *a priori* more effective seems therefore to be one of the most urgent tasks at the moment. In the special case of radiometals deposited in the respiratory tract, it would also be useful to test the effectiveness of a chelating agent administered in aerosol form. Exploratory tests by Semenov *et al.* (72) with this type of application have shown that EDTA is remarkably effective with radioyttrium. Both parenteral and oral administration of chelating agents seem to be contraindicated where radiometals are present in the digestive tract, since in some circumstances they could increase parenteral absorption of radiometals, and they may affect the effectivity of other materials administered for the purpose of inhibiting absorption (e.g., ion exchangers).

In conclusion we may deal briefly with the question of DTPA toxicity. As far as the decalcifying effects of Na_3-DTPA and the toxicity of single doses of $CaNa_3$-DTPA are concerned, exploratory tests (12, 23) suggest that the significant dosages are of the same order as those of EDTA. From the point of view of practical therapeutic use, the experiments with animals (27, 80) and the corresponding clinical trials (64, 74) have shown the exceptional importance of the nephrotoxic effects of *repeated* doses of chelating agents. Such effects are the limiting factor as regards the administration of chelating agents over as short a time as possible, which is desirable in itself. The question whether the nephrotoxic effect of DTPA differs quantitatively from that of EDTA still remains open. Since the exact cause of the kidney damage is still not clear (74), in our view, no *a priori* assertions can be made. Comparative toxicological tests with DTPA and EDTA and the determination of their therapeutic range are therefore desirable. Not until we have these results will we be justified in recommending the clinical use of DTPA without any restrictions.

REFERENCES

1. Anderegg, G., P. Nägeli, F. Müller and G. Schwarzenbach. *Helv. Chim. Acta* 42: 827, 1959.
2. Balabukha, V. S. and F. Ye. Fradkin. *Nakopleniye radioaktivnikh vechestv v organisme i ikh videleniye.* Moscow: Medgis, 1958.
3. Belyayev, Yu. A. *Med. Radiol.* 5, Nr. 2: 54, 1960.
4. Bjerrum, J., G. Schwarzenbach and L. G. Sillén. *Stability Constants. Pt. I: Organic Ligands.* London: The Chemical Society, 1957.
5. Bogucki, R. F. and A. E. Martell. *J. Am. Chem. Soc.* 80: 4170, 1958.
6. Catsch, A. *Strahlentherapie* 99: 290, 1956.
7. Catsch, A. *Naturwiss.* 44: 94, 1957.
8. Catsch, A. *Klin. Wchnschr.* 37: 657, 1959.
9. Catsch, A. and D. Kh. Lê. *Nature, London.* 180: 609, 1957.
10. Catsch, A. and D. Kh. Lê. *Strahlentherapie* 107: 494, 1957.
11. Catsch, A. and D. Kh. Lê. *Strahlentherapie* 107: 298, 1958.
12. Catsch, A., D. Kh. Lê and H. Melchinger. *Strahlentherapie* 106: 606, 1958.
13. Catsch, A. and H. Melchinger. *Strahlentherapie* 107: 437, 1958.
14. Catsch, A. and H. Melchinger. *Strahlentherapie* 108: 63, 1959.
15. Catsch, A. and H. Melchinger. *Strahlentherapie* 109: 561, 1959.
16. Chaberek, S., A. E. Frost, M. A. Doran and N. J. Bickwell. *J. Inorg. & Nuclear Chem.* 11: 184, 1959.
17. Chenoweth, M. B. *Pharmacol. Rev.* 8: 57, 1956.
18. Cohn, S. H., J. K. Gong and M. C. Fishler. *Nucleonics* 11, Nr. 1: 56, 1953.
19. Cohn, S. H., J. K. Gong and W. L. Milne. *A.M.A. Arch. Indust. Health* 14: 533, 1956.
20. Dudley, H. C. *J. Lab. & Clin. Med.* 45: 792, 1955.
21. Durham, E. J. and D. P. Ryskiewich. *J. Am. Chem. Soc.* 80: 4812, 1958.
22. Erleksova, Ye. V. *Med. Radiol.* 4, Nr. 8: 54, 1959.
23. Fahey, J. L., J. V. Princiotto, C. E. Rath and M. Rubin. *Clin. Res.* 8: 52, 1960.
24. Foreman, H. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 82.
25. Foreman, H. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 161.
26. Foreman, H. and C. Finnegan. *J. Biol. Chem.* 226: 745, 1957.
27. Foreman, H., C. Finnegan and C. C. Lushbaugh. *J.A.M.A.* 160: 1042, 1956.
28. Foreman, H., P. A. Fuqua and W. D. Norwood. *A. M. A. Arch. Ind. Hyg.* 10: 226, 1954.
29. Foreman, H., J. Post and B. C. Eustler. *Am. J. Roentgenol.* 79: 1071, 1958.
30. Foreman, H. and T. T. Trujillo. *J. Lab. & Clin. Med.* 43: 566, 1954.
31. Foreman, H., T. T. Trujillo, O. Johnson and C. Finnegan. *Proc. Soc. Exper. Biol. & Med.* 89: 339, 1955.
32. Foreman, H., M. Vier and M. Magee. *J. Biol. Chem.* 203: 1045, 1953.
33. Fried, J. F., E. H. Graul, J. Schubert and W. M. Westfall. *Atompraxis* 5: 1, 1959.
34. Fried, J. F., A. Lindenbaum and J. Schubert. *Fed. Proc.* 18: 229, 1959.
35. Fried, J. F., A. Lindenbaum and J. Schubert. *Proc. Soc. Exper. Biol. & Med.* 100: 570, 1959.
36. Fried, J. F., M. W. Rosenthal and J. Schubert. *Proc. Soc. Exper. Biol. & Med.* 92: 331, 1956.
37. Fried, J. F., J. Schubert and A. Lindenbaum. *A. M. A. Arch. Indust. Health* 20: 473, 1959.
38. Götte, H. *Z. Naturforsch.* 8 b: 173, 1953.
39. Graul, E. H., H. Hundeshagen and W. Schömer. *Strahlentherapie* 106: 391, 1958.
40. Hamilton, J. G. and K. G. Scott. *Proc. Soc. Exper. Biol. & Med.* 83: 301, 1953.
41. Harder, R. and S. Chaberek. *J. Inorg. & Nuclear Chem.* 11: 197, 1959.
42. Heller, H. J. and A. Catsch. *Strahlentherapie* 109: 464, 1959.
43. Hursh, J. B. *J. Pharmacol. & Exper. Therap.* 103: 450, 1951.
44. Hursh, J. B. *Proc. Soc. Exper. Biol. & Med.* 79: 210, 1952.
45. Ito, Y., S. Tsurufuji, S. Ishibashi, M. Ishidate, Z. Tamura and H. Takita. *Chem. & Pharm. Bull.* 6: 34, 1958.
46. Ito, Y., S. Tsurufuji, E. Murai, S. Ishibashi, M. Ishidate and Z. Tamura. *Chem. & Pharm. Bull.* 6: 92, 1958.
47. Ito, Y., S. Tsurufuji, M. Shikita and S. Ishibashi. *Chem. & Pharm. Bull.* 6: 288, 1958.
48. Kawin, B. *Experientia* 14: 373, 1958.
49. Kawin, B. and D. H. Copp. *Proc. Soc. Exper. Biol. & Med.* 84: 576, 1953.

50. KISIELESKI, W. E., W. P. NORRIS AND L. A. WOODRUFF. *Proc. Soc. Exper. Biol. & Med.* 77: 694, 1951.
51. KRIEGEL, H. AND H. MELCHINGER. *Atompraxis* 5: 425, 1959.
52. KROLL, H., S. KORMAN, E. SIEGEL, H. E. HART, B. ROSOFF, H. SPENCER AND D. LASZLO. *Nature, London* 180: 920, 1957.
53. KROLL, H. AND E. SIEGEL. *Fed. Proc.* 18: 267, 1959.
54. LANGHAM, W. H. *Health Phys.* 2: 172, 1959.
55. LINDENBAUM, A. AND J. F. FRIED. *Report Argonne Nat. Lab.*, January 1958.
56. LINDENBAUM, A. AND J. SCHUBERT. *Report Argonne Nat. Lab.*, December 1959.
57. MICHON, G. AND M.-J. GUILLOUX. *Compt. rend. acad. sci.* 248: 2039, 1959.
58. MOSKALEV, YU. I. *Med. Radiol.* 4, Nr. 1: 65, 1959.
59. NERURKAR, M. K. AND M. B. SAHASRABUDHE. *Proc. Indian Acad Sci.* 44: 73, 1956.
60. NEUMAN, W. F. AND M. W. NEUMAN. *The Chemical Dynamics of Bone Mineral.* Chicago: Univ. Chicago Press, 1958.
61. NEUMANN, K. H. AND E. HARBERS. *Arch. exper. Path. u. Pharmakol.* 217: 64, 1953.
62. NORWOOD, W. D., P. A. FUQUA AND B. C. SCUDDER. *Indust. Med.* 25: 135, 1956.
63. NORWOOD, W. D., P. A. FUQUA, R. H. WILSON AND J. W. HEALY. In: *Second United Nations Conference on Peaceful Uses of Atomic Energy*, Geneva: United Nations, 1958, vol. 23, p. 434.
64. ROSENTHAL, M. W., (editor) *Therapy of Radioelement Poisoning.* ANL-5584, 1956.
65. ROSENTHAL, M. W. In: *Symposium on Radioisotopes in the Biosphere*, University of Minnesota, Minneapolis, 1959.
66. ROSOFF, B., S. RITTER, K. SULLIVAN AND H. SPENCER. *Fed Proc.* 18: 131, 1959.
67. SCHUBERT, J. *Ann. Rev. Nuclear Sc.* 5: 369, 1955.
68. SCHUBERT, J. *Atompraxis* 4: 393, 1958.
69. SCHUBERT, J. AND J. F. FRIED. *Nature, London* 185: 551, 1960.
70. SCHUBERT, J. AND H. WALLACE. *J. Biol. Chem.* 183: 157, 1950.
71. SEMENOV, D. I. *Trudy Inst. Biol. Akad. Nauk S.S.S.R.*, Ural'. Filial. 9: 4, 1957.
72. SEMENOV, D. I. AND I. P. TREGUBENKO. *Trudy Inst. Biol. Akad. S.S.S.R.*, Ural'. Filial. 9: 59, 1957.
73. SEMENOV, D. I. AND I. P. TREGUBENKO. *Biokhimiya* 23: 59, 1958.
74. SEVEN, M. J. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 10.
75. SMITH, V. H. *Nature, London* 181: 1792, 1958.
76. SOWBY, F. D. AND D. M. TAYLOR. *Brit. J. Radiol.* 33: 342, 1960.
77. SPENCER, H., J. SAMACHSON AND D. LASZLO. *Proc. Soc. Exper. Biol. & Med.* 97: 565, 1958.
78. SPODE, E. AND F. GENSICKE. *Strahlentherapie* 111: 476, 1960.
79. THILO, E. *Angew. Chem.* 67: 141, 1955.
80. TREGUBENKO, I. P. *Doklady Akad. Nauk S.S.S.R.* 110: 872, 1956.
81. VOLF, V. *Nature, London* 184: 1401, 1959.

DISCUSSION

Radioelement removal by chelating agents: application of mass action laws and other factors

JACK SCHUBERT
Argonne National Laboratory, Lemont, Illinois

DR. CATSCH'S PAPER was so very complete and extensive that I will comment on only a few selected portions. First, I would like to comment on Dr. Bell's statements concerning the use of exponential and log functions. Sometimes the impression is given that a log-log function is better than an exponential. Certainly, the log-log is worthwhile to use because one may get a straight line covering several half-lives. But the exponential can be used and is fundamentally just as good, especially if one has a model based on discrete compartments. However, when one uses an exponential function, one must be very careful to specify the time interval over which it is applied.

I would like to correct one thing in the discussion of Dr. Kehoe. I might have given the impression that I think no chelating agent should be used for nonradioactive metals. This is not the point. My point is that some agents can work and do work quite well, such as penicillamine, it is just that eventually none of them will work as well as a substance which fixes the toxic metal at the site of its deposition. In this connection, it is illuminating to cite results of treatment of beryllium poisoning with two identical chelating agents, except that one has a sulfonic acid group. If you give these drugs four hours after administration of beryllium, both compounds cause the same marked increase of excretion of beryllium but one protects and one does not. The difference lies in the fact that the less water soluble form inactivates a greater fraction of the toxic metal *within* the tissues.

Dr. Catsch mentioned the failure of BAL in radioelement removal. The reason for this is that practically all the radioelements of importance do not react with sulfur ligand atoms, namely, plutonium, alkaline earths and rare earths. Under physiological conditions there is no stable complex formed, and, therefore, except for polonium, BAL will be ineffective.

When applying mass action equations to estimate the relative effectiveness of chelating agents, there are often too many unknown factors to evaluate. However, one can use a very simple experimental set up involving ultrafiltration under conditions closely approximating physiological to obtain reliable data in a short time (4). Instead of using chelating agents in time-consuming animal experiments, one can compare dozens of chelating agents in a few hours. Without exception, the results obtained in these ultrafiltration experiments match those *in vivo* (1).

One convenient way to test the effect of chelating agents, instead of adding the radioelement directly to blood serum withdrawn from the animal, is to inject the radioelement into the animal and then withdraw samples of blood containing the radioelement. To the blood one then can add known amounts of chelating agent and measure the ultrafilterability. In this way one knows the radioelement is present in the actual state present in the animal—an especially important point with hydrolyzable elements (1, 5).

There is another point that Dr. Catsch mentioned on which he did not have the time to go into detail. In his Figure 1, he found no correlation between the removal from tissues of various elements such as lead, mercury and strontium and the ratio of the formation constant of the metal ion to that of calcium. Actually, this relationship can be applied most accurately only to a group of cations with closely related properties or the same cation with different chelating agents. For example, if one is comparing a series of closely related metal ions whose binding constants for the chelate and other ions, such as hydroxide, are very similar, then one gets very good correlation. In other words, if one is comparing the removal of different rare earth radioelements with a chelating agent, one finds good correlation on a plot of the degree of removal from the tissue with the formation constants. The reason is that the range of variation of the constants for hydroxide formation, $M(OH)^{++}$, is quite small, for example—a factor of ten between the lightest and heaviest rare earths. Hence, it can be expected that the differences in protein binding and other interactions largely cancel out if you compare the same metal ion with different chelating agents (2). However, when comparing elements widely scattered in their hydrolytic reactions such as mercury, cerium and strontium, one then has to insert a few more additional terms as a minimum, such as the hydroxylation constants. In the case of Dr. Catsch's data, when one inserts the constant for hydroxylation for mercury and the other elements in his Figure 1, the inconsistencies then are removed.

Some time ago (2), I published quantitative formulations relating the efficiency of removal of radioelements to their formation constants with chelating agents and other ligands. From mass action considerations—omitting nonequilibrium processes—it is possible to estimate the fraction of radioelement bound by the ligands involved. In my previous publication (2), the expressions I derived for those cases in which the effects of hydrolysis and protein interaction were considered were incorrect. I have been meaning to correct them for sometime and this is a good time to do so. The equations given below include corrections of *equations XIII* and *XIV* given in Reference 2. I wish to acknowledge the cooperation of Dr. Norman C. Li of Duquesne University in the derivation of the corrected forms.

Consider a simple exchange reaction:

$$CaV + M = MV + Ca \qquad (1)$$

where V represents the molar concentration of chelating agent, Ca that of calcium ion, and CaV and MV the 1:1 chelates. Charges on the ions are omitted for simplicity. It has been shown (2) that the ratio of M chelated to the free or unchelated form is given by the expression:

$$R_{MV} = \frac{(MV)}{(M)} = \frac{K_{MV}}{K_{CaV}} \times \frac{(Vt)}{(Ca)} \qquad (2)$$

where K_{MV} and K_{CaV} are the respective formation constants, V_t = total V = V − CaV; it has also been shown that (Ca) × $K_{CaV} \gg 1$ in serum.

When the hydroxylated forms, represented symbolically by MOH, are present, then the equilibria are:

$$CaV + (M + MOH) = (MV + MOHV) + Ca \qquad (3)$$

which leads to an extended form of *equation 2*:

$$R = \frac{(MV + MOHV)}{(M + MOH)} = \frac{K_{MV} + K_{MOH}K_{MOHV}(OH^-)}{K_{CaV}(1 + K_{MOH}(OH^-))} \times \frac{V_t}{(Ca)} \qquad (4)$$

The equilibrium constant for (3) is given by the expression:

$$K = \frac{(MV + MOHV)(Ca)}{(CaV)(M + MOH)} = \frac{K_{MV} + K_{MOH}K_{MOHV}(OH^-)}{K_{CaV}(1 + K_{MOH}(OH^-))} \qquad (4')$$

where

$$K_{MOHV} = \frac{(MOHV)}{(MOH)(V)}$$

When both MOH and MP are considered, where P represents the protein binding constituents, then:

$$CaV + (M + MOH + MP) = (MV + MOHV) + (Ca + CaP) \qquad (5)$$

Let

$$R' = \frac{\text{M bound to V}}{\text{M not bound to V}} = \frac{(MV + MOHV)}{(M + MOH + MP)}$$

then

$$R' = \frac{K_{MV} + K_{MOH}K_{MOHV}(OH^-)}{K_{CaV}[1 + K_{MOH}(OH^-) + K_{MP}(P)]} \times \frac{V_t}{(Ca)} \qquad (6)$$

In his Figure 1, Dr. Catsch plotted the per cent removal of several radioelements *vs.* the log $\frac{(MV)}{(CaV)}$. If instead of log $\frac{(MV)}{(CaV)}$ the expression,

$$\log \frac{K_{MV} + K_{MOH}K_{MOHV}(OH^-)}{K_{CaV}(1 + K_{MOH}(OH^-))}$$

as given in *equation 4'* is used, then the apparent inconsistencies are eliminated. For example, in the case of mercury and EDTA where $Hg(OH)_2$ is the stable form, ($K_{MOH} = 10^{22}$) and ($K_{MOHV} \sim 10^8$) are present at pH 7. Then, from *equation 4* it is found that $R \sim 10^{-4}$ or not much different than for Sr, despite the fact that $K_{HgV} = 10^{22}$ while $K_{SrV} = 10^9$.

It is interesting to note that *equation 4* also indicates that EDTA will not influence mercury poisoning because under physiological conditions the fraction of mercury bound is insignificant. Experimentally, it has been shown that the acute toxicity of mercury administered as the mercury EDTA chelate is no different from unchelated mercury. Of further interest is the fact seen from *equation 6* that the influence of protein binding is relatively unimportant when the hydrolytic constants are large.

It is often difficult for a chelating agent to reach the deposited radioelements. For this reason the use of esterified derivatives which would be hydrolyzed at the intracellular pH is an important development. In line with this work, it is also important to ascertain the removal of radioelements from the intracellular fractions of tissues as well as from the whole tissue. There are many examples known in which a chelating agent has effected a net removal of a radioelement from an organ, such as the liver, and yet the net amount deposited in the mitochondria actually has increased. When one considers that one of the radiosensitive sites in the cell may be associated with the mitochondria, then it is important to ascertain the degree of removal of a radioelement from specific cell fractions as Dr. Catsch has so well demonstrated.

While it is possible to remove many radioelements from the body, it is also important to demonstrate that such removal also reduces the tumor incidence which follows much later. In long term experiments in progress at Argonne, we have found that the removal of plutonium effected by DTPA treatment given three days after injection of Pu causes a marked decrease in bone tumor incidence. These experiments, however, are not considered complete until all the histological evidence is gathered. In addition, we are also investigating whether DTPA removes the Pu from specific histological sites or from diffuse areas.

Dr. Foreman has asked me to comment on the strontium problem. In a previous publication (3) I evaluated the potential value of chelating agents for the removal of radiostrontium by the use of *equation 1*. It turned out that there were severe limitations since the Sr chelates are always more weakly bound than the corresponding Ca chelates. This does not mean that it is impossible to remove Sr but that it is necessary to use high concentrations maintained for long periods in the blood in order to remove appreciable amounts of Sr. The problem is analogous to an extraction column and the calculations for estimating the efficiency of removal are analogous to those in which the concept of HETP—the height of a theoretical plate—is employed in countercurrent operations. In the biological system, one can utilize the time necessary for the volume of blood in the body to be cleared through the kidney as a single "plate." In any event, in order to remove appreciable strontium, it is necessary that R in *equation 1* be about 0.1 *and* that the strontium can be contacted by the chelating agent. The latter condition means that to a large extent only the Sr in the soft tissues and bloodstream can be removed to a significant extent by prolonged treatment with a chelating agent.

Decalcification procedures can remove Sr from bone but only in experimental animals where the degree of decalcification attained can be very high. In man, however, this is not a practical procedure. While many possibilities exist for removing strontium from man (3) no practical ones appear for removing Sr already fixed in the bone. Such possibilities as the use of nonradioactive strontium carriers can be shown to be impractical by means of the mass action equations.

REFERENCES

1. LINDENBAUM, A. AND J. SCHUBERT. *Nature, London* 187: 575, 1960.
2. SCHUBERT, J. *Ann. Rev. Nuclear Sc.* 5: 369, 1955.
3. SCHUBERT, J. *Atompraxis* 4: 393, 1958.
4. SCHUBERT, J. AND E. E. CONN. *Nucleonics* 4: 2, 1949.
5. SCHUBERT, J. AND J. F. FRIED. *Nature, London* 185: 551, 1960.

DR. RAY: Just so we don't end on a pessimistic note, it is possible to mobilize strontium from the bone even though one can't do it readily with EDTA. This goes back to some experiments that Dr. Aub (Aub, J. C., R. D. Evans, D. M. Gallagher and D. M. Tibbetts. *Ann. Int. Med.* 11: 1443, 1938) did years ago on the mobilization of radium from bone using a rachitogenic diet and subsequent experimental studies by Dr. Copp (Copp, D. H., J. G. Hamilton, D. C. Jones, D. M. Thompson and C. Cramer. In: *Metabolic Interrelations*, trans. Third Conf., edited by E. C. Reifenstein. New York: Macy, 1951, p. 226, and Copp, D. H., J. G. Hamilton, D. C. Jones, D. M. Thompson and C. Cramer. Univ. Calif. Rad. Lab., Berkeley. *UCRL* 1464: 10, 1951.). Our experience (Ray, R. D. and I. Lyon. *J. Bone & Joint Surg.* 42-A: 832, 1960.) would confirm Dr. Copp's that the low calcium–low vitamin D diet is relatively ineffective in contrast to a low phosphate diet. There are, however, three limitations to the low phosphate diet. One is that the increased rate of mobilization of Sr^{90} from the body may cause increased damage to the kidney. The second is that there is a delay of about one week between the onset of the diet and increased mobilization, a week in which the isotope (Sr^{90}) may become firmly accreted by the bone. And the third is the damage to the organism by the low phosphate diet. If a rat is kept on a low phosphate diet for 60 days or more the thorax collapses and the animal dies of respiratory failure; but between 7 and 60 days the body burden of Sr^{90} may be reduced 80 per cent.

DR. COTZIAS: I would like to ask Dr. Catsch to correlate his two studies, chelation therapy with loading of animals with stable strontium to promote the excretion of Sr^{90}.

DR. CATSCH: Both principles are, in the case of radiostrontium, slightly effective. The deposition in the skeleton by isotopic dilution, i.e., stable strontium chloride, can be reduced by about 20 per cent; by the most effective chelator, the monoether polyamino acid (BADE, BAETA), by 10–15 per cent. The combination of the two principles, that means the administration of the strontium chelate, effects a reduction by 40 to 50 per cent, so that the actions of isotopic dilution and the chelator seem to be additive.

DR. HERTA SPENCER (Montefiore Hospital, New York): I should like to make some remarks in regard to the enhancement of radiostrontium excretion as well as to the usefulness of certain chelating agents for decontamination. I should also like to mention some studies which have some bearing on those just presented a few minutes ago. In terms of radiostrontium decontamination or removal in man, a very simple method can be used, namely, the intravenous administration of stable calcium or its combination with orally administered ammonium chloride. Either of these two agents lead to increased urinary excretion of calcium and of radiostrontium. This method is so far the most effective in decreasing the radiostrontium body burden (Spencer, H., J. Samachson, B. Kabakow and D. Laszlo. *Clin. Sc.* 17: 291, 1958). I do not, off hand, remember the figures, but I should like to mention one particular case studied under controlled dietary conditions in which 44 per cent of the dose of radiostrontium was retained in the control study while the combined use of calcium gluconate and of ammonium chloride decreased the body retention to less than 10 per cent. These results were obtained when the enhancing agents were given at the time the tracer was administered, but this combined regimen or the use of intravenous calcium gluconate alone, or of orally administered ammonium chloride are effective also as late as two weeks after the intravenous administration of the Sr^{85} tracer (Spencer, H., D. Laszlo and J. Samachson. *Fed. Proc.* 18: 149, 1959, and Spencer, H., A. Feldstein and J. Samachson. *Fed Proc.* 19: 351, 1960).

This would indicate that maybe a larger amount of radiostrontium than heretofore believed is still available in the soft tissue for removal, since it is not easily understandable how an appreciable amount of radiostrontium could be removed from bone at that time. In regard to chelating agents, I agree, as Dr. Schubert has mentioned, that EDTA is not effective in removing radiostrontium. In fact, the sodium salt of EDTA not only does not increase but inhibits the excretion of Sr^{85} (Spencer, H., J. Samachson and D. Laszlo. *Proc. Soc. Exper. Biol. & Med.* 97: 565, 1958). The calcium salt of EDTA changes the excretion of Sr^{85} only very slightly. Similarly DTPA has also only a slight but insignificant effect. However, calcium BAETA which has been previously used by Dr. Kroll in experiments in rats (Kroll, H. and E. Siegel. *Fed. Proc.* 18: 267, 1959) has been found to be effective in increasing the Sr^{85} excretion in man from three to five fold in the "early" phase, the highest excretion was noted on the first day of administration of calcium BAETA (Spencer, H., A. Feldstein and J. Samachson. *Fed. Proc.* 19: 351, 1960).

In addition I should like to mention that this chelating agent was still effective two weeks after the administration of Sr^{85}. Chelating agents are also effective in the removal of other radioisotopes, for instance, we have been able to remove yttrium (Rosoff, B., S. Ritter, K. Sullivan and H. Spencer. *Fed. Proc.* 18: 131, 1959), and scandium (Rosoff, B., E. Siegel, G. Williams and H. Spencer. *Fed. Proc.* 19: 354, 1960) from the body very effectively with EDTA and DTPA. DTPA was more effective than EDTA. Similarly, zinc can be very effectively removed from the body by using EDTA or DTPA, the main pathway of excretion being the kidney. Zinc could be removed as late as 60 days after administration of Zn^{65} tracer (Spencer, H. *Ann. New York Acad. Sc.* 88: 435, 1960). I was very interested to hear that Dr. Schubert and his group now also use ultrafiltration as an indication of the effectiveness of chelates. I should like to point out that Lewin, Rosoff and others from our laboratory in 1956 (Lewin, R., B. Rosoff, H. E. Hart, K. G. Stern and D. Laszlo. In: *Advances in Radiobiology*. Edinburgh: Oliver, 1956, p. 298) and Rosoff *et al.* in 1958 (Rosoff, B., R. Lewin, H. E. Hart, G. L. Williams and D. Laszlo. *Arch. Biochem. & Biophys.* 78: 1, 1958) have published data on the ultrafilterability of a series of 11 yttrium chelates and have found that the filterability correlated very well with the stability constants and also with the magnitude of urinary excretion in man. Therefore, if the *in vitro* filterability of the metal chelate is high this is an indication that this particular chelate would be effective also for the removal of the particular isotope.

Some effects of chelating agents on tryptophan metabolism in man[1]

J. M. PRICE[2]

Department of Surgery, University of Wisconsin Medical School, Madison, Wisconsin

THIS DISCUSSION WILL BE CONCERNED with the effects of Na_2EDTA on the metabolism of tryptophan along the tryptophan-niacin pathway in patients with various diseases. The same subject has been considered in detail in previous publications from these laboratories (9-11, 13, 14) and no attempt will be made here to present a complete summary of this topic.

The pathway under consideration involves the conversion of tryptophan to several aromatic amines, aminophenols, quinoline and pyridine derivatives (Fig. 1). As discussed previously (9–11, 13–15) several members of the vitamin B complex play an important role in the conversion of tryptophan to niacin (Fig. 2). It is of particular interest that the vitamin niacin in the form of triphosphopyridinenucleotide is essential for the maintenance of the pathway (5). There is no evidence that the other products of this pathway have any essential function in metabolism.

A study of Figures 1 and 2 makes it possible to predict that a functional deficiency of pyridoxine might be expected to result in the accumulation of kynurenine, 3-hydroxykynurenine and their acetyl derivatives. When patients were placed on courses of the pyridoxine antimetabolites, isoniazid and deoxypyridoxine, and given loading doses of L-tryptophan there was a considerable increase in the urinary excretion of these metabolites (12). Patients ingesting deoxypyridoxine also excreted increased quantities of kynurenic and xanthurenic acids (12) which indicates that this drug had relatively less effect on the transamination reactions which result in the formation of these quinoline derivatives (Figs. 1 and 2). On the basis of studies of this type it was concluded that other patients who excrete abnormally large quantities of kynurenine, hydroxykynurenine, their acetyl derivatives, kynurenic acid or xanthurenic acid following ingestion of a loading dose of tryptophan have a disorder of tryptophan metabolism which is the result of a functional pyridoxine deficiency (9).

Patients have been evaluated with respect to their ability to metabolize tryptophan in the following manner (9–15). One or two 24 hour urine collections were obtained following which 2.0 gm of L-tryptophan were administered. The urine collected before and after the ingestion of tryptophan was analyzed for its content of these metabolites. The results have been compared with data obtained in a similar manner from subjects with no known disease. Subtraction of the basal level of the urinary metabolite from the level present after the ingestion of the tryptophan supplement gives the response to the loading dose of tryptophan expressed as the quantity excreted in excess of the basal level. Data so collected from patients with no known disease yields what has been called the normal pattern of urinary metabolites. The different pattern of metabolites excreted in disease has then been referred to as an abnormal pattern of metabolites; and such patterns show evidence of both qualitative and quantitative abnormalities (9). A summary of some of the results that have been obtained (9) has been presented in Figure 3.

Examination of the results in Figure 3 reveals evidence of a functional pyridoxine deficiency in each of the seven clinical conditions represented in that there is an elevation of the average levels of excretion of kynurenine and hydroxykynurenine and other metabolites are often excreted in increased quantities. Some of the procedures which have been found to correct these abnormal metabolic patterns will now be considered.

ISONIAZID AND DEOXYPYRIDOXINE

Patients ingesting these drugs were given supplements of the vitamins which are known or suspected to be required for the metabolism of tryptophan by this pathway (Figs. 1 and 2). It was found that supplements of thiamine, riboflavin and niacin had little effect on the metabolism of tryptophan by patients ingesting isoniazid or deoxypyridoxine while pyridoxine supplementations completely corrected the metabolic abnormality (12). Thus the action of isoniazid and deoxypyridoxine on tryptophan metabolism in man may

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[2] American Cancer Society-Charles S. Hayden Foundation Professor of Surgery in Cancer Research.

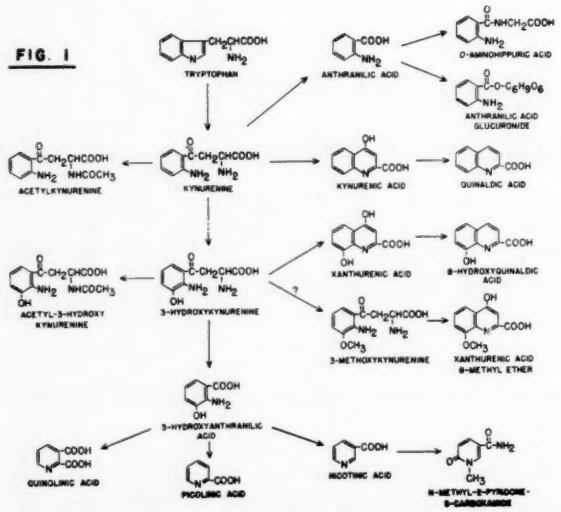

FIG. 1. An outline of the pathway from tryptophan to niacin showing some of the products and intermediates of the pathway.

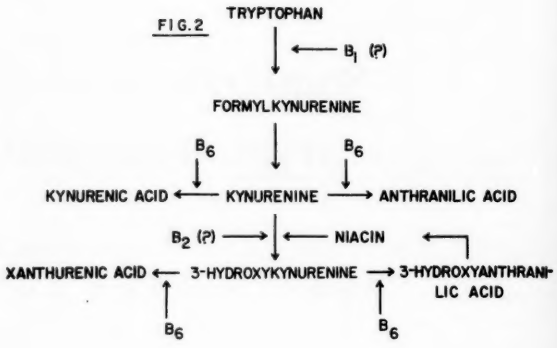

FIG. 2. The site of action of several members of the vitamin B complex on the metabolic pathway from the essential amino acid tryptophan to the vitamin niacin. The roles of thiamine (B_1) and riboflavin (B_2) have not been clearly established so their position in the pathway has been indicated by a question mark. As indicated previously (9) the action of pyridoxine (B_6) in this pathway appears to be well established.

be explained by the known anti-pyridoxine action of these drugs.

PREGNANCY

Supplements of the B-complex vitamins without pyridoxine had little or no effect on the metabolism of tryptophan by pregnant women (2). The addition of pyridoxine to the supplement resulted in considerable improvement in the metabolic pattern in pregnancy (2), but it would appear that factors other than the B-complex may be involved in the production of the unique pattern of urinary tryptophan metabolites seen in this condition (Fig. 3). These results will be presented in more detail elsewhere.

CANCER

The cancer patients studied (Fig. 3) were those with carcinoma of the urinary bladder. The abnormal pattern of urinary tryptophan metabolites seen in about half of the patients with bladder cancer may be almost completely corrected by supplementation with pyridoxine (3, 9).

PORPHYRIA AND SCLERODERMA

Patients with porphyria, scleroderma and bladder cancer excreted patterns of tryptophan metabolites which were essentially identical (Fig. 3). However, the patterns of metabolites excreted by patients with scleroderma or porphyria were often refractory to pyridoxine supplementation (9–11, 13, 14) while the patients with bladder cancer responded almost completely to the administration of this vitamin (3, 9). Thus, the patients

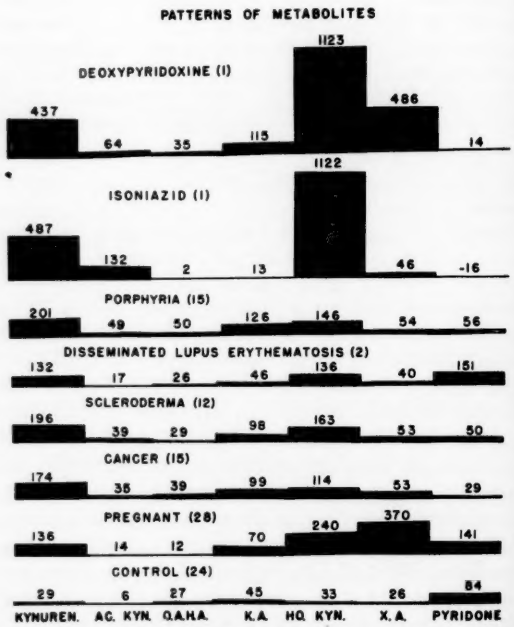
PATTERNS OF METABOLITES
DEOXYPYRIDOXINE (1)
437 64 35 115 1123 486 14
ISONIAZID (1)
487 132 2 13 1122 46 -16
PORPHYRIA (15)
201 49 50 126 146 54 56
DISSEMINATED LUPUS ERYTHEMATOSIS (2)
132 17 26 46 136 40 151
SCLERODERMA (12)
196 39 29 98 163 53 50
CANCER (15)
174 36 39 99 114 53 29
PREGNANT (28)
136 14 12 70 240 370 141
CONTROL (24)
29 6 27 45 33 26 84
KYNUREN. AC. KYN. O.A.H.A. K.A. HO. KYN. X.A. PYRIDONE

FIG. 3. Pattern of metabolites excreted by a group of subjects with no known diseases (controls) as compared with the patterns of metabolites excreted by patients with various clinical conditions (9). The results represent averages from the number of subjects indicated by the figure enclosed by parenthesis. The height of each bar is proportional to the number above the bar and this number indicates quantity of metabolite excreted in micromoles above the basal level following ingestion of 2 gm of L-tryptophan. KYNUREN. = kynurenine; AC. KYN. = acetylkynurenine; O.A. H.A. = *o*-aminohippuric acid; K.A. = kynurenic acid; HO. KYN. = hydroxykynurenine; X.A. = xanthurenic acid; and PYRIDONE = *N*-methyl-2-pyridone-5-carboxamide.

with scleroderma and porphyria metabolized tryptophan as though they were deficient in pyridoxine but they failed to respond as expected to supplementation with the vitamin.

That patients with porphyria and scleroderma both appear to be afflicted with some abnormality of mineral metabolism has been emphasized previously (8, 13, 14, 16). Furthermore, many patients with porphyria or scleroderma show clinical improvement when they are treated with metal-binding agents (8, 16). During such treatment, there is usually a marked diuresis of zinc, copper or both and the patients frequently metabolize tryptophan normally after chelation therapy. In porphyria and scleroderma, therefore, Na_2EDTA has been observed to have pyridoxine-like activity. An example of the comparative effects of Na_2EDTA and pyridoxine on the tryptophan metabolism of a patient with scleroderma is shown in Figure 4.

This young female with advanced scleroderma metabolized tryptophan in a very abnormal manner before treatment (Fig. 4). On her first admission to the hospital she was given 5 doses of 3 gm of Na_2EDTA and at the end of the period she metabolized tryptophan as shown on the left half of Figure 4 (+EDTA). Pyridoxine hydrochloride was then administered with 10 additional doses of Na_2EDTA and her responses to a loading dose of tryptophan became more nearly normal (+EDTA+B_6). Several months later, on her next admission to the hospital, she again metabolized tryptophan in a very abnormal fashion before treatment, as shown on the right half of Figure 4. She was given supplements of pyridoxine and restudied (+B_6) before receiving another course of 15 doses of Na_2EDTA along with pyridoxine. At the end of the course of therapy she responded to tryptophan loading (+EDTA+B_6) much as she had on the first admission. Comparison of the effects of Na_2EDTA and pyridoxine on the tryptophan metabolism of this patient reveals that these effects were very similar. The combination of Na_2EDTA and pyridoxine appeared slightly more effective than either preparation alone. The patient showed clinical improvement following each course of therapy. Other examples of this phenomenon have been reported (10, 11, 13, 14).

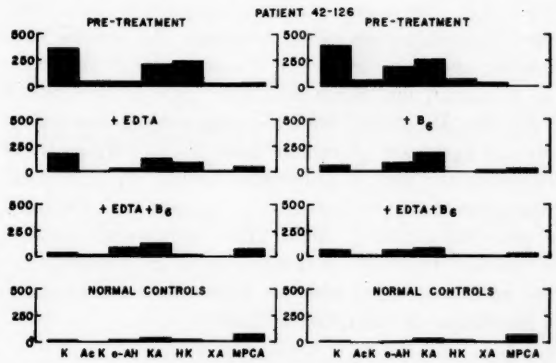

FIG. 4. Effects of Na_2EDTA (EDTA) and pyridoxine (B_6) on the tryptophan metabolism of a patient with scleroderma (11). The scale indicates micromoles of metabolite excreted after the ingestion of 2 gm of tryptophan. This patient was studied under various conditions and the results are compared with the average response of 30 controls (normal controls) to the same dose of tryptophan. Further details are given in the text. K = kynurenine; AcK = acetylkynurenine; *o*-AH = *o*-aminohippuric acid; KA = kynurenic acid; HK = hydroxykynurenine; XA = xanthurenic acid; MPCA = *N*-methyl-2-pyridone-5-carboxamide.

DISCUSSION

It should be emphasized that patients with many clinical conditions metabolize tryptophan normally. Thus patients with diabetes, psoriasis, asthma, atopic dermatitis, thyrotoxicosis, and various neurological conditions metabolized tryptophan essentially as did the controls (2, 13). Most of the clinical conditions in which there is some associated abnormality of tryptophan metabolism show a pattern of urinary tryptophan metabolites which suggests a pyridoxine deficiency (Fig. 3). It is not remarkable that in several of these conditions the excretion of tryptophan metabolites becomes normal when supplements of pyridoxine are administered. It was not anticipated, however, that in patients with porphyria and scleroderma Na_2EDTA would produce an effect similar to that expected of pyridoxine.

The mechanism by which Na_2EDTA may produce a pyridoxine-like effect on tryptophan metabolism is not clear. However, since the most characteristic property of Na_2EDTA is its ability to bind certain trace metals, it is very likely that this drug produces its pyridoxine-like action as a result of some effect on the trace metals. It has been shown that Na_2EDTA produces a diuresis of copper, zinc or both in patients with scleroderma (16) or porphyria (8, 13). However, Perry and Schroeder (7), Clarke *et al.* (4) and Boyle *et al.* (1) have reported the development of skin lesions resembling those found in pyridoxine deficiency in patients with atherosclerosis during prolonged treatment with large doses of Na_2EDTA. These lesions were reported to improve or disappear when pyridoxine supplements were administered. Therefore, there is evidence that under certain conditions Na_2EDTA may have anti-vitamin B_6 activity (1, 4, 7) or under other conditions it may have pyridoxine-like activity (11).

The administration of Na_2EDTA to subjects with an essentially balanced supply of minerals might be expected to create a mineral imbalance of some type. Such patients then might show evidence of a functional deficiency of pyridoxine just as patients with porphyria and scleroderma, who also have some abnormality of mineral metabolism and have an associated disorder of tryptophan metabolism which resembles that seen in pyridoxine deficiency. In patients with porphyria and scleroderma, the Na_2EDTA may tend to correct a mineral imbalance thereby allowing the pyridoxine to function more effectively. Thus, if a mineral imbalance does result in some impairment of the function of pyridoxine, one can more readily understand these effects of

Na_2EDTA. The administration of Na_2EDTA could then show pyridoxine-like or anti-vitamin B_6 activity depending upon whether or not it was correcting or creating a mineral imbalance (11, 13).

Metzler, Ikawa and Snell (6) proposed that the active form of pyridoxal phosphate may be a complex with the substrate and a polyvalent cation. It appeared reasonable to suggest (10, 11, 13, 14) that an imbalance of polyvalent cations might affect pyridoxine activity at this site. However, it remains to be determined just how an imbalance of minerals might affect the function of pyridoxine in the intact animal.

SUMMARY

Many patients with scleroderma or porphyria metabolized tryptophan as though they had a functional deficiency of pyridoxine. These patients showed some biochemical improvement in their tryptophan metabolism when supplemented with pyridoxine but intravenous Na_2EDTA appeared to possess as much pyridoxine activity as pyridoxine itself. It is proposed that the pyridoxine-like effect of Na_2EDTA on the tryptophan metabolism of patients with scleroderma or porphyria was the result of a partial correction of an imbalance of polyvalent cations which appears to be present in these patients.

REFERENCES

1. Boyle, A. J., J. J. Jasper, H. McCormick, M. Kosai, D. McCann, J. Goodwin, N. E. Clarke and R. E. Mosher. *Bull. schweiz. Akad. med. Wissensch.* 13: 408, 1957.
2. Brown, R. R. and J. M. Price. Unpublished data.
3. Brown, R. R., J. M. Price, E. J. Satter and J. B. Wear. *Acta Unio internat. contra cancrum.* 16: 299, 1960.
4. Clarke, N. E., C. N. Clarke and R. E. Mosher. *Am. J. M. Sc.* 229: 142, 1955.
5. De Castro, F. T., J. M. Price and R. R. Brown. *J. Am. Chem. Soc.* 78: 2904, 1956.
6. Metzler, D. E., M. Ikawa and E. E. Snell. *J. Am. Chem. Soc.* 76: 648, 1954.
7. Perry, H. M., Jr. and H. A. Schroeder. *Am. J. Med.* 22: 168, 1957.
8. Peters, H. A., S. Woods, P. L. Eichman and H. H. Reese. *Ann. Int. Med.* 47: 889, 1957.
9. Price, J. M. *Univ. Michigan M. Bull.* 24: 461, 1958.
10. Price, J. M. *Proceedings of the Twelfth Research Conference, American Meat Institute Foundation*, University of Chicago, 1960, p. 47.
11. Price, J. M. and R. R. Brown. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 179.
12. Price, J. M., R. R. Brown and F. C. Larson. *J. Clin. Invest.* 36: 1600, 1957.
13. Price, J. M., R. R. Brown and H. A. Peters. *Neurology* 9: 456, 1959.
14. Price, J. M., R. R. Brown, J. G. Rukavina, C. Mendelson and S. A. M. Johnson. *J. Invest. Dermat.* 29: 289, 1957.
15. Price, J. M., J. B. Wear, R. R. Brown, E. J. Satter and C. Olson. *J. Urol.* 83: 376, 1960.
16. Rukavina, J. G., C. Mendelson, J. M. Price, R. R. Brown and S. A. M. Johnson. *J. Invest. Dermat.* 29: 273, 1957.

GENERAL DISCUSSION

Dr. Philips: What is known about the renal mechanism involved in the excretion of these metabolic end products? The amounts excreted in 24 hours may represent but small fractions of the total processed through the kidney. If, by chance, metal(s) were involved in reabsorption of the metabolites, the presence of an exogenous chelating agent in tubular urine might easily disturb such a process, resulting in increased excretion of the metabolites.

Dr. Price: Very little is known about the mechanism by which these tryptophan metabolites are cleared by the kidney. We have previously discussed (Price, J. M. *Univ. Michigan M. Bull.* 24: 461, 1958) the possible explanations for the fact that patients with various clinical conditions excrete from 10 to 25 times the normal amounts of these metabolites after the ingestion of a 2 gm loading dose of tryptophan. Most of the available evidence suggests that in these patients the pathway from tryptophan to niacin is partially blocked at various points, resulting in accumulation and subsequent excretion of increased quantities of these intermediary metabolites. It appears considerably less likely that altered renal mechanisms could explain the increased excretion of these metabolites by the patients.

[Shortly after Dr. Price began his paper on Friday morning, the projector lamp burned out, causing an interruption of a few minutes.

Dr. Hastings filled in the time with remarks on the number of times metals have been identified as participants in known enzymatic reactions of intermediary metabolism. To illustrate his remarks, he displayed two intermediary metabolism charts prepared in 1960 by H. J. Sallach and R. W. McGilvery. He had marked and counted the number of times a metal appeared on the charts and put the following data on the blackboard:

Metal	Times Appearing
Mg	58
Mn	2
Zn	3
Mo	3
Fe	8
K	2
Total	76

His remarks, partially recorded, were as follows:]

Dr. Hastings: "As you all doubtless recognize, these are the pathways of metabolism that our cells are undergoing at the present time. My count of the number of reactions involved exceeds 280. Every place you see a red square, you see a metal that is required for a reaction, or rather I should say, if you were to hit a red square at random, 58 times out of 76, you'd hit magnesium. Your chances of hitting zinc are, by my count, 3 to 76 and iron, about 8—manganese 2."

Trace minerals, chelating agents and the porphyrias[1]

HENRY A. PETERS
University of Wisconsin Medical School, Madison, Wisconsin

INTEREST IN DISTURBANCES of porphyrin metabolism was stimulated in 1953 when we observed a near fatal case of acute hepatic porphyria manifesting severe quadraplegic motor neuropathy, bulbar symptoms, hypertension and psychosis who responded dramatically to 2,3-dimercaptopropanol (BAL) after all other measures, including ACTH therapy and barbiturate withdrawal, failed to halt her downhill course (23).

A careful screening program at University Hospitals directed at the identification of patients manifesting abnormal porphyrin metabolism has produced a series of over 75 patients who demonstrated abnormal porphyrin excretion during periods of clinical symptomatology. We have reported previously on results of chelation therapy with BAL and ethylenediaminetetraacetic acid (EDTA) in the therapy of 45 porphyria patients in which we noted a favorable therapeutic effect in approximately two-thirds of the patients (23–27). Treanor and Rupe (34) and McCabe (16) report on the therapeutic effectiveness of chelation in single severely involved cases of acute porphyria, while Painter (21) described the therapeutic benefit in three out of four acute porphyria patients treated in this manner. Galambos and Peacock (5) mention five patients who manifested porphobilinogenuria as well as elevation of urinary lead levels who responded "dramatically" to calcium sodium EDTA and described a 50 year old patient with extremely high levels of urinary lead excretion (thought due to contamination) who seemed to react adversely to EDTA administration. Wirtschafter, Turner and Dow (41) report unsatisfactory results of chelation with EDTA in a 16 year old porphyric with recurring status epilepticus. Whereas we have found that diphenylhydantoin (Dilantin) is poorly tolerated by the porphyric patients (23, 26, 27), and have excluded it and other barbiturates wherever possible during chelation efforts, Dilantin was continued concomitantly with their chelation efforts using only parenteral EDTA. Paul and Thyresson (22) as well as Schrumpf (31) report chronic porphyria cases manifesting cutaneous symptoms that benefited from BAL therapy, while Woods, Peters and Johnson (42) have reported on a case of chronic porphyria with porphobilinogenuria that was helped by oral administration of calcium disodium versenate ($CaNa_2EDTA$).

Watson (38) divides porphyria into two major forms. First, the porphyria erythropoetica (congenital porphyria) in which the metabolic error resides in the bone marrow with onset in infancy of mutilating light sensitive dermatitis and, second, the hepatic porphyrias which he subdivides into acute cutanea tarda or chronic, mixed, and latent forms. Acute hepatic porphyria inherited as a Mendelian dominant is characterized by recurrent gastrointestinal, psychiatric and neurological symptomatology occurring alone or in combination. Whereas erythropoetica porphyria is rare, hepatic porphyria appears to be far more common than heretofore suspected. Hepatic cutanea tarda porphyria (chronic porphyria) is manifested by the late onset of photosensitive cutaneous lesions while mixed porphyria incorporates a combination of the cutaneous and acute varieties in which cutaneous, neurological, psychiatric and abdominal symptomatology appear in varying combinations. A latent form of the disorder exists in which the patient may be clinically asymptomatic.

The genetic aspects of the porphyrias have been given intensive study by Dean and Barnes (4), Waldenstrom (37) as well as by Watson and co-workers (38, 39). Dean and Barnes' South African type of porphyria is apparently a common disorder among the European population of South Africa and is genetically different from the Swedish or Waldenstrom type. The South African variety is inherited as a Mendelian dominant gene and is not sex-linked. Acute attacks occur most commonly in females between the ages of 16 and 50. Acute attacks are precipitated by drugs, especially barbiturates. The acute attacks resemble the acute attack in the Swedish type (37) and in the past were usually fatal. Light sensitivity was found in some of the male members of the family, and there was a high excretion of porphobilinogen during the attack with normal excretion of porphobilinogen as the patient recovered. In remission these patients showed an increase of fecal

[1] We are especially indebted to Dr. F. L. Kozelka and Mrs. Wanda McCarty who guided our laboratory work, and also to David Stuiber, Barbara McCann and Lyra Hekmatpanah for their assistance. Dr. H. H. Reese, Dr. P. L. Eichman and Dr. S. Johnson likewise collaborated in the clinical study. This work has been aided by National Institutes of Health grant B-1943(C1).

porphyrin, present also during the acute attack, while the excretion of porphobilinogen tended to normalize in the quiescent phase.

Using Dean's method (4) of estimating an increase in fecal porphyrins (consisting of both protoporphyrin and coproporphyrin), we have been screening our neurological and psychiatric population so as to identify likely patients for further study. We then utilize the quantitative estimation of porphobilinogen and delta-amino levulinic acid developed by Granick and Vanden Schreik (8), and also Mauzerall and Granick (15), plus qualitative determinations of coproporphyrin and uroporphyrin when clinical signs and symptoms suggested the diagnosis. Since the acute porphyria patients often will demonstrate porphobilinogen and uroporphyrin excretion intermittently, multiple Watson-Schwartz tests for urinary porphobilinogen may be necessary to rule out this metabolic error when the clinical inventory shows symptoms suggestive of acute porphyria. Moreover, cases of chronic porphyria manifesting actinic or photosensitive, often bullous, skin lesions and ease of abrasion of exposed dermal surface areas will demonstrate uroporphyrin in the urine while porphobilinogen may never be demonstrated at times even when the patient develops neurological and psychiatric manifestations provoked by the administration of barbiturates or sulfa drugs (mixed porphyria).

We have used the term, "paraporphyric" (25, 26), to classify those patients clinically resembling the known porphyric patients often with a history of intolerance to paint, sulfonamides and barbiturate drugs, but in whom excretion of urinary uroporphyrin and porphobilinogen cannot be demonstrated. The paraporphyric will manifest at times an increased fecal "protoporphyrin" excretion (Dean's test), at times an elevated delta-amino levulinic acid in the urine, and at times, when symptomatic, an increase in urinary zinc or copper. By labeling these patients as "paraporphyrics" and treating them as porphyrics, we automatically avoid the use of barbiturates and sulfa drugs, which would, if they were porphyrics in a latent phase, precipitate a possible fatal crisis. A similarity of the abnormal tryptophan tolerance studies reported by Price, Brown and Peters (28) in the porphyric group during the acute stages of the disorder is mirrored in some of our paraporphyrics that we have studied in this manner, suggesting a close relationship. We would join others who have called attention to the similarity of lead intoxication and the symptoms observed in cases of acute porphyria (22, 37). We would add to this the often striking similarity between cases of acute porphyria and the signs and symptoms seen in other intoxications due to arsenic, mercury, acute delirium tremens, Wernicke's encephalopathy and in some of the collagen disorders.

SYMPTOMATOLOGY

The world literature calls attention to a tremendous variety of gastrointestinal, dermatological, psychiatric and neurological symptomatology observed in patients suffering from this disorder. In this regard, we would especially direct your attention to the descriptions of Goldberg (6), Dean and Barnes (4), Waldenstrom (37), Watson and Larson (39), Brunsting (3) and others. We have, ourselves, summarized and given extensive case reports on the symptomatology presented by our porphyric patients and the effects of chelation therapy (23–27, 42).

The symptomatology which we have observed may be summarized as follows, recognizing that symptoms will vary from patient to patient, and especially during their longitudinal history to the extent that a patient with abdominal colic or back pains, after using sulfa or barbiturate drugs, may develop profound neurological and psychiatric symptoms in the form of seizures, psychosis, peripheral neuropathy, amblyopia, etc.:

Gastrointestinal symptoms in some patients were entirely lacking, the symptoms being entirely confined to the neurological and psychiatric realm. Gastrointestinal symptoms, when present, included recurrent abdominal or back pain, usually without muscle guarding and often accompanied by nausea, emesis, intermittent diarrhea (at times bloody) or severe constipation with frequent fecal impactions. Although lower abdominal pain has been thought to be most typical, the colic frequently mimicks gall bladder disease, pancreatitis, or even lower chest pathology.

Urinary incontinence or retention was not infrequent and anuria, especially after the administration of sulfonamide drugs including sulfisoxazole (Gantrisin), has been observed. One patient had cystoscopy performed more than a dozen times because of a suspected primary kidney disturbance. On several occasions, the urinary complaints were thought secondary to conversion hysteria or a schizophrenic psychosis until the metabolic alterations indicative of porphyria were noted. Repeated surgical intervention was not an infrequent finding, one woman having had a series of twelve major abdominal operations. Such a history of polysurgery should always be cause for consideration of the diagnosis of acute intermittent porphyria. Contrary to popular belief, the burgundy wine or brownish urine described as typical in textbooks may never be mentioned by the acute porphyric except under careful questioning while many patients' urine did not change to brown, red or black, even after exposure to sunlight and acidification, despite the presence of increased porphobilinogen in the urine. At times, nurses and patients described the urinary odor as particularly offensive.

Neurological signs and symptoms included bulbar involvement in the form of dysphagia, diplopia, anisocoria, dysarthria, facial paralysis and laryngeal palsies. Tracheotomy was often necessary in patients showing early evidence of respiratory involvement. There was a high incidence of grand mal seizures; Jacksonian or psychomotor seizure activity also was seen and accompanied by focal electroencephalographic localization. Headaches of migraine type were noted. Two patients manifested

homonomous hemianopsia which, in one patient, alternated from one side to another and normalized on recovery of the patient. Status epilepticus of severe degree was often recorded. Both cerebellar ataxia and extrapyramidal symptoms have been noted (in the absence of phenothiazine derivatives), and intolerance to diphenylhydantoin medication has been noted in some of the patients treated with this drug for seizure activity. A surprising number of patients had diabetic relatives.

Psychiatric symptoms were present in over half of our patients. These symptoms consisted of toxic psychosis with bizarre, catatonic-like excitement, misidentification, déjà vu, auditory and sometimes visual hallucinosis, resembling in this regard, patients with acute delirium tremens. Several patients showed a tendency to confabulate in a manner identical to a Korsakoff alcoholic psychosis. The level of consciousness at times ranged from torpor to coma. Well over a dozen patients have manifested a schizophrenic-like psychosis which could not be differentiated from the classical schizophrenia by Rorschach test on independent psychiatric evaluation. It is our definite feeling that a porphyric schizophrenic group exists as a clinical entity. The erroneous diagnosis of hysteria often had been attached to our patient group prior to the development of more severe and obvious neuropsychiatric symptomatology. Depressive or schizo-affective symptoms likewise were noted.

A large number of patients presented peripheral neuropathy including complete tetraparesis in eight, paraplegia in three and monoplegia in one. These more severe neurological symptoms were precipitated often by sulfonamide or barbiturate medication, especially the quick-acting barbiturates such as thiopental (Pentothal). Not infrequently, one obtained a history of prior intolerance to barbiturate drugs, especially the quick-acting barbiturates, or chronic use of phenobarbital over periods ranging from several months to several years. The paresis was as often distal in onset as proximal, and frequently was accompanied by recurrent myoclonic jerks and asynchronous muscle contractions (especially against resistance) and a spreading excitation of the muscles of the body seen when a single extremity was moved against resistance, not unlike that seen in strychnine intoxication. This heightened motor irritability often existed despite complete tetraplegia, and deep tendon reflexes were sometimes retained despite complete paralysis. Surprisingly, sensory alterations were usually completely absent despite severe motor weakness. Muscle and joint pain was often seen during the onset and recovery phase of the neuropathy. The pain, however, was so fleeting and vague or poorly localized as to suggest hysteria initially, reflecting most probably an altered level of consciousness. We noted one patient who sustained a fracture dislocation of the shoulder who did not complain of her pain until after her level of consciousness was improved following BAL treatment. Another patient had sustained a severe fracture of the dorsal spine in a fall from her bed with resultant paraparesis and a sensory level. Her altered level of consciousness, however, obscured this clinical feature until more careful neurological examination and x-rays had been obtained. Laminectomy had been performed on two patients for a pseudo-cord tumor.

A malar flush over the butterfly area was observed resembling lupus, especially in the acute and mixed hepatic porphyrics during exacerbations. A dusky metallic complexion, increased melanosis or violaceous hues often were seen in the mixed and chronic porphyrics over light-exposed areas, while some of the acute porphyrics likewise showed these features. The chronic and mixed porphyrics manifested skin which abraded easily and actinic and photosensitive bulli were seen.

A careful inventory often revealed a history of intolerance to oil-base paints and chemical solvents in approximately one-third of the patients. In several of our patients, use of oil-base paints and, in one, exposure to gasoline products in his work seemed to play a part in precipitating acute neurological and psychiatric syndromes. Also high on the inventory was a history of intolerance to Pentothal in previous operative procedures, as well as intolerance to sleeping medications (most often barbiturate derivatives). Where intolerance to Pentothal existed, similar intolerance to other barbiturates likewise was observed; although of all the barbiturates, the slow acting ones were best tolerated. A marked weakening effect on prolonged sunbathing was noted; and one patient had her first attack of acute porphyria precipitated after falling asleep in the sun and developing a severe case of sunburn.

METHOD OF TREATMENT

Dimercaptopropanol (BAL) was used as a 10 per cent solution in 20 per cent benzyl benzoate in peanut oil. Dosage ranged from 50 to 1200 milligrams per 24 hours in divided doses by the intramuscular route. Therapy varied from four to sixty days consecutively. Although it has seldom been indicated to continue chronic injections of BAL, we have given, in several cases, one to three injections of 100–300 milligrams weekly, for as long as two years in one instance. Other than a questionable related transient erythema multiforme and several instances of sterile abscess formation at the injection site, no toxic effects of BAL or sodium EDTA and its calcium chelate have been encountered. Calcium disodium EDTA was used initially for intravenous administration, while our most recent intravenous studies have been with disodium EDTA.

Intravenous dosage of EDTA averaged 2.5 grams in a 24 hour period. Although we have gone as high as 5 grams in the past several years, we seldom exceeded a dosage level of 2.5 grams per day. The disodium versenate was administered over a three to four hour period in 1000 milliliters of 5 per cent glucose in water. Administration of this compound was continued over a two to five day period, depending upon the clinical response. In several cases, we have given courses of daily

administration for periods up to several weeks. Where indicated, calcium disodium EDTA in .5 gram tablets has been used in subacute phases of the condition, the daily dose varying from .5 to 2 grams per day. The choice of chelating agent depended in part on a history of lead exposure; EDTA compounds were used preferentially in the initial phases of treatment when urinary lead excretion levels were borderline or when the patient had had known exposure to lead. Particularly in the male, we have observed a profound lead diuresis during the initial courses of therapy; and we suspect that BAL administration in the presence of large available lead stores may be hazardous unless the patient has been given calcium disodium versenate in a preliminary course of treatment.

After the diagnosis was made or suspected barbiturates, sulfonamides, alcohol, heavy metals, exposure to oil-base paints, solvents and sunbathing were studiously avoided. Cases of chronic porphyria with porphobilinogenuria developed abdominal colic when placed on Chloroquine (42). Linden, Newcomer, and Chapman (14) have reported a case of porphyria precipitated by Chloroquine administration for chronic discoides erythematosus, while another report refers to progesterone induced porphyria as reported by Levit, Nodine, and Perloff (13). Whereas we have utilized cortisone in patients with severe oliguria for short periods of time with benefit (23, 27), the use of steroids in the porphyric patient especially with neurological and psychiatric symptoms may entail considerable hazard (20, 36). Chlorpromazine, meperidine (Demerol), paraldehyde, azacyclonol (Frenquel), chloral hydrate and intravenous procaine were used very occasionally, although chlorpromazine, meperidine and Frenquel were seldom necessary during chelation therapy or thereafter.

In addition to chelating agents, we wish to emphasize that exceptional nursing care, tracheotomy and careful attention to fluid and electrolyte balance must be credited in helping to bring about clinical recovery in our most severe cases. During the recovery phase, we also have utilized powdered glycine in dosage of 1 dram three times a day plus vitamin B_{12} in a controlled study program to determine whether glycine, acting as a precursor of porphobilinogen would enhance the patient's performance. Vitamin B_{12} has been shown to reduce the urinary excretion of porphyrins (1).

A total of fifty-eight patients have now been treated with chelating agents, including forty-two acute intermittent porphyrics, five mixed hepatic porphyrics, two chronic porphyrics and nine paraporphyrics. Thirty-two of the forty-two acute intermittent porphyrics, five of the mixed porphyrics, nine of the paraporphyrics and two of the chronic porphyrics seemed to benefit from chelation schedules. Of the acute porphyrics not showing a favorable response, one of them died with periarteritis nodosa which had been diagnosed by biopsy prior to chelating efforts. This patient had also been treated previously and had shown profound deterioration with alternating ACTH and cortisone prior to and concomitantly with a final attempt to improve his state with chelation. Another severe acute intermittent porphyric who developed complete tetraplegia and bulbar paralysis died of a staphylococcal bacteremia despite what seemed to have been an initial favorable response to chelation efforts. Two acute hepatic porphyrics with predominantly abdominal symptoms, and with associated addiction, committed suicide sometime after discharge from the hospital. Nineteen of the acute hepatic porphyrics and two of the mixed porphyrics were in very critical condition from advanced neuropsychiatric symptomatology prior to chelation efforts despite prior ineffectual use of ACTH in five cases. All but four of this group seemed to have benefited from chelation therapy, with complete recovery from a tetraparesis having been noted in eight patients. We have reported previously one tetraparetic patient who derived benefit from ACTH after BAL treatment seemed inadequate (23). One patient, previously reported with dorsal transverse myelitis and peripheral motor neuropathy of the upper extremities, recovered function of her arms and hands after chelation therapy; but the myelopathy was not changed until the last two years when she has shown an unexpected progressive return of some sensory and some motor function in her lower paralyzed extremities (23). We wish to note again that the recovery rate of porphyric neuropathy is dependent upon the degree to which axon cylinders have been altered, in addition to the known demyelinization that takes place.

It is extremely important that physical therapy be continued through a convalescence that may take many months. However, it should be mentioned that we have noted immediate gains in strength following BAL or EDTA compounds in eight patients whose recovery from severe peripheral neuropathy prior to chelation treatment had reached a prolonged plateau. Of great interest was the rapid improvement in schizophrenic and other psychiatric symptoms frequently noted after initiation of chelation efforts. Seven porphyric schizophrenics have shown reversal of psychotic symptoms on chelation therapy alone, while a number of others with combined neurological and psychiatric symptoms recovered also on chelation regimens. In the absence of addiction, those with predominantly abdominal colic frequently showed a favorable response to chelation although those in whom addiction was suspected were especially difficult to evaluate. Oral maintenance regimens with calcium disodium EDTA have been helpful in aborting later attacks, although additional courses of intravenous EDTA have, at times, been necessary. The addition of glycine in dosage of one dram three times daily to oral chelation with EDTA has, during convalescence from residual motor weakness, seemed to play a strengthening role over and above that noted with EDTA alone. Vitamin B_{12} also in some instances has been employed during the convalescent phase and the patients have reported a strengthening effect. In many instances the use of chlorpromazine, which is helpful in symptomatic therapy (17, 18, 32), has not been necessary, although we have on occasion employed chlorpromazine as an alternative

regimen for comparative purposes. We have been struck by the "Parkinsonizing" effect of the phenothiazine derivatives in some of these patients. The use of prochlorperazine (Compazine) in two patients provoked a severe trismus and marked asthenia in one, and severe weakness in the other when it was employed during a recurrent attack of abdominal colic.

During the recovery phase of peripheral neuropathy, pain at times has been severe, requiring use of intravenous procaine for additional relief, although generally these efforts were not necessary, excepting during the initial recovery phase. It should also be mentioned that several patients with severe peripheral neuropathy seemed especially sensitive to the depressant effects of chlorpromazine to the extent that 25 mg of chlorpromazine in one instance produced a transient period of apnea and deep coma. Monaco and associates (18) noted inordinate somnolence in their patients and relatives of patients after small doses of chlorpromazine.

The course of acute porphyria is notoriously variable and we must recognize that our therapeutic results represent primarily our clinical impressions. We have tried to compare the results of chelation efforts with the clinical picture of the patient when maintained on merely supportive regimens with or without the addition of phenothiazine derivatives. Nevertheless, the frequently prompt response following chelation therapy in some cases with severe neurological and psychiatric symptomatology suggests strongly that chelation played a prominent role in the results observed. For pertinent case histories demonstrating the effect of chelation in porphyria, we refer you to our previous publications and the work of others (16, 21–27, 31, 34, 41, 42).

Peripheral neuropathy in several instances showed improvement within a matter of hours; and there was further objective evidence of improved mental status, cessation of recurrent seizures, clearing of aphasia disturbance and recovery from coma. A long period of follow up of this patient group, going back as far as six years, impresses us with the treatability of this condition even when the patient has been severely involved from a neurological or psychiatric standpoint. In contrast, we have a series of four patients who were not treated by chelation; in this group one treated with cortisone because of arthralgic symptoms died shortly after dismissal from the hospital, and the three others have required a prolonged period of hospitalization on more conservative measures. The seizures that have occurred in our porphyric series of patients have frequently resolved during chelation therapy and we have found it best to avoid the use of Dilantin and other anticonvulsants with the exception of paraldehyde and rectal Avertin in bringing about seizure control. Dilantin especially has seemed to be poorly tolerated and this agent was utilized by Wirtschafter *et al.* (41) concomitantly with their chelation efforts. In patients such as theirs whose seizures were controlled only with Dilantin and who continued to have a wide spread organic encephalomyelopathy, we have been inclined to treat with more prolonged administration of dimercaptopropanol and exclusion of Dilantin and barbiturates wherever possible.

The two cases of chronic porphyria, one treated with initial intravenous versenate and a maintenance regimen of oral EDTA, and the other treated with oral EDTA alone, have both registered satisfactory improvement in clinical status including a decided decrease in photosensitive disfiguring skin lesions, the period of observation extending back for five years in the case of one, and for three years in the other. The patient previously reported (42) has been able to tolerate sun exposure and even ethanol and his skin lesions, ease of abrasion of the skin and abdominal symptoms have not recurred since his chelation treatment began in 1956.

DISCUSSION

Our attempts to explain the seeming effectiveness of chelation therapy in aborting the neuropsychiatric and gastrointestinal symptoms of acute intermittent porphyria, mixed porphyria, as well as in influencing the photosensitive dermatological lesions in chronic porphyria focused our attention on the increased urinary zinc, and at times copper, excretion which seemed to parallel symptomatology in some of our cases during exacerbations of the condition better than did the excretion of abnormal urinary porphyrins (24–27). An increase in copper excretion was noted especially in the chronic and mixed porphyrics also during periods of clinical symptomatology (26). The values for copper and zinc diuresis were especially high in those patients showing peripheral neuropathy, psychosis or seizures with the patients demonstrating primarily splanchnic or visceral symptoms showing less high or at times normal values.

Urinary zinc was high in twelve of eighteen acute porphyric patients prior to chelation therapy. Copper excretion was elevated in six of the chronic and mixed porphyrics studied, six of whom have now been chelated. Increased urinary excretion of both copper and zinc was noted before chelation in seven of the acute patients. The values for elevated copper excretion before chelation ranged from two to five times the norm and for zinc from two to thirty-six times the normal. The zinc and/or copper diuresis noted in the porphyric patients prior to chelation and grossly augmented during chelation therapy, tended to approach normal values during the recovery phase despite continuance of chelation and often continued elevation of abnormal porphyrin excretion. This normalization of copper and zinc urinary excretion seems typical of the porphyric in a state of remission. Where the urinary zinc and copper is derived from is speculative, although it could include increased absorption of these compounds from the alimentary canal as is seen regarding copper in Wilson's disease or diversion of excretion routes from the fecal to the urinary tract. While Nesbitt has postulated that a zinc deficit was responsible for the symptoms in porphyria (19), the augmentation of urinary zinc excretion during chelation therapy along with clinical improve-

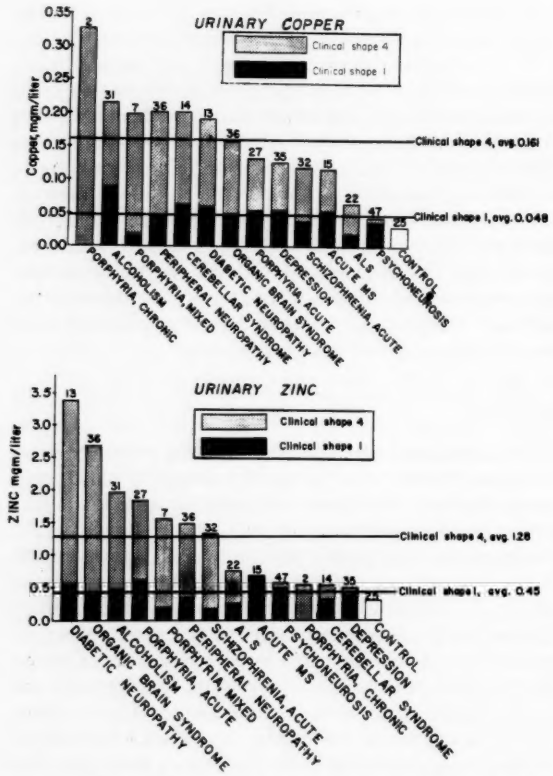

FIG. 1. Urinary copper and zinc excretion in various clinical states.* †

* Clinical shape four represents the average values for patients showing active clinical signs and symptoms. Clinical shape one represents the levels during periods of remission or recovery. High values for copper and zinc were likewise seen in periarteritis nodosum and intoxication with lead, arsenic and mercury.

† Heavy metal determinations were calculated on a per liter basis, because the excretion of copper and zinc seems to parallel that of lead rather than representing a constant excretion factor as for example with urea or creatinine. Diuresis due to increased fluid intake in a normal individual can, if carried to extremes, bring the urinary excretion of copper, zinc and lead up to a pathological level if calculated on a per 24 hour basis. The excretion of these compounds on a per liter basis is remarkably constant during a 24 hour period.

ment would suggest that this is not true. Another possibility, however, would be that a less available cation may be the true offender, or that the process of chelation itself brings about a re-distribution of these cations throughout the body that is more favorable to normal metabolic processes. Certainly balance studies are in order to rule out the possibility of increased alimentary absorption when copper and zinc diuresis are noted. However, it is our impression that an elevation of urinary copper and zinc excretion in the porphyric as well as in several other diseases with severe neurological and psychiatric problems is often proportional to the severity of the neurological and psychiatric clinical symptomatology and reflects an increased inability of the body to handle these and perhaps other cations.

Gunther (9) had recognized that urinary porphyrins were often combined with a metal and Watson and Schwartz (40) noted the zinc metal complexing of uroporphyrin and coproporphyrin. Watson and Larson (39) later called attention to the zinc complex being relatively larger in amount in a case of porphyria showing relapse. For this reason, we had postulated that the metabolic block in acute intermittent porphyria, which presumably lies beyond the porphobilinogen stage, might be due to an excess retention of zinc and/or copper or perhaps some other cations. We have noted that an excess of zinc is said to inhibit the enzymatic activity of lactic and alcohol dehydrogenase, and also delta-amino levulinic dehydrase, while insulin, ACTH and equine gonadotropin activity are retarded on addition of zinc (25–27). The zincuria, which occurs in the acute hepatic porphyric, and the increased copper and sometimes increased zinc excretion observed in the mixed and chronic forms of the disorder could represent Nature's attempt to rid the body of toxic cations by means of increased excretion of porphyrin metabolites in combination with zinc (or other cations) as a last desperate effort to mobilize these porphyrin substances for the needed purposes of chelation.

As suggested by Watson and Larson (39), Klüver's discovery (11) of the regular occurrence of small amounts of coproporphyrin in the central nervous system of warm-blooded animals, and the tentative identification as the type III isomer (identified by Blanshard (2) as the type III isomer) together with the increased excretion of this isomer in poliomyelitis, lead intoxication, as well as in porphyria, lead one to question whether the urinary porphyrin may be derived, at least in part, from the central nervous system. Solomon and Figge (33) have pointed out that both coproporphyrin III and protoporphyrin may be extracted more abundantly from the peripheral nerves than from the central nervous system of pigs. They further postulate that the porphyrins might be involved in the process of conduction of nerve impulses. Granick and Gilder (7) suggest that coproporphyrin may serve as a regulator of oxygen consumption by living cells, and Watson and Larson (39) add that nervous tissue might produce and liberate it much more rapidly under certain circumstances. Keilin (10) has suggested that cytochrome might be the parent substance of coproporphyrin and Klüver pointed out that the areas relatively rich in cytochrome were poor in coproporphyrin, or devoid of it entirely and *vice versa.*

We would then suggest that, in porphyria, coproporphyrin III and protoporphyrin might be withdrawn from the central, peripheral and sympathetic nervous system for purposes of chelation thus, after a time, depriving the nervous system of important metabolites with the resultant production of the very multitudinous symptoms seen in this disorder. Likewise, assuming that the porphyrins and cytochrome in the central nervous system and peripheral nerves require constant replenishment or exchange, the block above the porphobilinogen stage possibly due to excess zinc or copper could prevent

the formation and maintenance of the necessary protoporphyrin, coproporphyrin III and cytochrome necessary to maintain normal function of the nervous system. Alteration in blood-brain barrier could account for a spread of porphyric symptoms from the peripheral and autonomic nervous system to the central nervous system, while barbiturate or sulfonamide series of drugs might well provide the "monkey wrench" on a hepatic level or even locally within the central nervous system where often, even in remission, there is not infrequently increased fecal and urinary diversion of porphyrins which, in the normal individual, are utilized for the production of usable metabolites.

Price, Brown and Peters (28) have noted an elevation of kynurenic acid or kynurenine, or both, after a loading dose of tryptophan during acute stages of acute, chronic and mixed porphyria resembling the tryptophan metabolic pattern seen in patients with scleroderma by Rukavina, Mendelson, Price, Brown and Johnson (29, 30). Some patients in our paraporphyric group shared this anomaly of tryptophan metabolism, as did some patients with acute schizophrenia (possibly paraporphyrics). Price, Brown and Peters (28) have noted that some of these tryptophan metabolites are, in themselves, excellent metal-binding agents.

The similarity of porphyric symptoms to those seen in lead intoxication where an increased excretion of coproporphyrin III also is seen, have paralleled our experience with cases of lead intoxication where we have observed, in addition to increased excretion of coproporphyrin, an elevation of delta-amino levulinic acid, and an increased excretion of copper and zinc in the urine, also frequently in direct proportion to the severity of the peripheral and central nervous system involvement. Galambos and Peacock (5) have referred to six porphyrics in which lead excretion values were pathological prior to chelation efforts in which five out of six patients responded to therapy. While they caution on the use of chelating agents in treating the porphyric, one might better conclude that acute porphyria is an infinitely more serious illness when complicated or precipitated by lead intoxication in which the lead chelate of EDTA may be relatively unstable.

Patients with mercury and arsenic intoxication also show a tremendous increase in urinary copper and zinc. The basis for the use of BAL has been the fact that this substance replaces sulfhydryl groups known to be exhausted in arsenic and probably in other heavy metal intoxications. If, in these conditions where zincuria and cupruria are observed, there has been a similar exhaustion of the body's natural chelating ability (chelation pool) such as sulfhydryl groups, diversion of porphyrin substances and tryptophan metabolites could well represent alternative adaptive mechanisms which, however, seriously embarrass the metabolism of the central and peripheral nervous system. We have noted also the similarity of symptoms in porphyria to those seen in delirium tremens, Wernicke's encephalopathy, and other organic brain syndromes. It is well known that ethanol often has a deleterious effect on the chronic and mixed porphyric, in particular those showing neuropsychiatric symptomatology. Vallee (35) has demonstrated zincuria in the alcoholic patient with cirrhosis, and we would like to add our observation of a tremendous cupruria as well as zincuria in many alcoholic patients suffering from delirium tremens, Wernicke's encephalopathy, and neuropathy. Vallee has postulated that it is withdrawal of zinc from the liver that may give rise to the development of cirrhosis and the symptoms in the alcoholic. We have, however, utilized EDTA and BAL in some of our alcoholics with severe involvement of the central and peripheral nervous system and observed, as in the porphyric, augmentation of zinc and copper excretion that did not deteriorate, but seemed to improve, the patient's clinical status. In those patients with porphyria as well as in those with lead, mercury, arsenic intoxication and in the alcoholic, we have observed a normalization of copper and zinc excretion as the patient's symptomatology improved.

SUMMARY

Symptoms and signs as seen in acute, chronic and mixed porphyric patients are summarized and we call attention again to the existence of a porphyric schizophrenic patient group. Elevation of urinary zinc in the acute porphyric as well as increased elevation of urinary copper, particularly in the mixed and chronic hepatic porphyric patients suggests the possibility that excess zinc, copper or other cations might be inducing a block of several metallo-enzyme systems. Exhaustion of certain portions of the body's natural chelating defenses and subsequent depletion of coproporphyrin III from the central and peripheral nervous system for purposes of chelation might be responsible for the symptoms observed in this disorder. Artificial chelation with EDTA or BAL could act by replenishing the depleted chelation reserves of the body, thus restoring more normal metabolism. The use of glycine and oral EDTA has seemed helpful in more chronic recovery phases of the disorder. Chelation therapy with BAL, EDTA or both, has seemed helpful in thirty-two of forty-two intermittent porphyrics, five out of five mixed porphyrics, in two of the chronic porphyrics, and in nine of the paraporphyric group. Not to be discounted has been the importance of maximal nursing care, tracheotomy, attention to electrolyte balance, and avoidance of barbiturates, sunbathing, steroids, and heavy metals in the more seriously involved patients.

ADDENDUM TO CONCLUSIONS

We call attention to the similarity of symptoms observed in the porphyric patient and the symptoms produced by heavy metal intoxication as well as in cases of alcoholism. In these conditions, as well as others, in which there is acute, evolving disturbance of the central nervous system, an increased urinary excretion of copper and/or zinc may be seen, as in the porphyric, which seems to be proportionate to the clinical symptomatology. Normalization

of these urinary metal values seems to occur on recovery of these patients just as in the porphyrics.

The recent observations reported by Rudi Schmid in the *New England J. of Med.*, August 25, 1960, concerning an epidemic-like outbreak of chronic and probably mixed porphyria in Turkey due to exposure of the population to hexachlorobenzene-treated grain suggests that exposure to certain noxious chemicals can induce clinical porphyria perhaps without the genetic error.

REFERENCES

1. ANTENER, I. AND H. BERGER. *Internat. Ztsche. Vitaminforsch.* 27: 233, 1957.
2. BLANSHARD, T. P. *Proc. Soc. Exper. Biol. & Med.* 82: 512, 1953.
3. BRUNSTING, L. A., H. L. MASON AND R. A. ALDRICH. *J.A.M.A.* 146: 1207, 1951.
4. DEAN, G. AND H. D. BARNES. *Brit. M. J.* 1: 298, 1958.
5. GALAMBOS, J. T. AND L. B. PEACOCK. *Ann. Int. Med.* 50: 1056, 1959.
6. GOLDBERG, A. *Quart. J. Med.* 28: 183, 1959.
7. GRANICK, S. AND H. GILDER. *Science* 101: 540, 1945.
8. GRANICK, S. AND H. G. VANDEN SCHRIECK. *Proc. Soc. Exper. Biol. & Med.* 88: 270, 1955.
9. GÜNTHER, H. *Ergebn. Allg. Path.* 20: 608, 1922.
10. KEILIN, D. *Proc. Roy. Soc. London ser. B.* 98: 312, 1925.
11. KLÜVER, H. *Science* 99: 482, 1944.
12. KLÜVER, H. *Proc. 1st Internat. Neurochem. Symposium*, Magdalen College Oxford. New York: Acad. Press, 1954.
13. LEVIT, E. J., J. H. NODINE, AND W. H. PERLOFF. *Am. J. Med.* 22: 831, 1957.
14. LINDEN, I. H., C. G. STEFFEN, V. D. NEWCOMER AND M. CHAPMAN. *California Med.* 81: 235, 1954.
15. MAUZERALL, D. AND S. GRANICK. *J. Biol. Chem.* 219: 435, 1956.
16. MCCABE, E. S. *Am. Pract. & Digest Treat.* 6: 878, 1955.
17. MELBY, J. C., J. P. STREET, AND C. J. WATSON. *J.A.M.A.* 162: 174, 1956.
18. MONACO, R. N., R. D. LEEPER, J. J. ROBBINS AND G. L. CALVY. *New England J. Med.* 256: 309, 1957.
19. NESBITT, S. *J.A.M.A.* 124: 286, 1944.
20. OLSON, O. C. AND M. H. STILES. *Ann. Int. Med.* 41: 357, 1954.
21. PAINTER, J. T. AND E. J. MORROW. *Texas J. Med.* 55: 811, 1959.
22. PAUL, K. G. AND N. THYRESSON. *Acta dermat.-venereol.* 34: 403, 1954.
23. PETERS, H. A. *Neurology* 4: 477, 1954.
24. PETERS, H. A. *Dis. Nerv. System* 17: 177, 1956.
25. PETERS, H. A. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 190.
26. PETERS, H. A., P. L. EICHMAN AND H. H. REESE. *Neurology* 8: 621, 1958.
27. PETERS, H. A., S. WOODS, P. L. EICHMAN AND H. H. REESE. *Ann. Int. Med.* 47: 889, 1957.
28. PRICE, J. M., R. R. BROWN AND H. A. PETERS. *Neurology* 9: 456, 1959.
29. PRICE, J. M., R. R. BROWN, J. G. RUKAVINA, C. MENDELSON AND S. A. M. JOHNSON. *J. Invest. Dermat.* 29: 289, 1957.
30. RUKAVINA, J. G., C. MENDELSON, J. M. PRICE, R. R. BROWN AND S. A. M. JOHNSON. *J. Invest. Dermat.* 29: 273, 1957.
31. SCHRUMPF, A. *Acta. med. scandinav.* 145: 338, 1953.
32. SEIDE, M. J. *New England J. Med.* 258: 630, 1958.
33. SOLOMON, H. M. AND F. H. FIGGE. *Proc. Soc. Exper. Biol. & Med.* 97: 329, 1958.
34. TREANOR, W. J. AND C. E. RUPE. *Henry Ford Hosp. Med. Bull.* 3: 162, 1957.
35. VALLEE, B. L., W. E. WACKER, A. F. BARTHOLOMAY AND F. L. HOCH. *New England J. Med.* 257: 1055, 1957.
36. VINE, S., H. M. SHAFFER, G. PAULEY AND E. J. MARGOLIS. *Ann. Int. Med.* 47: 834, 1957.
37. WALDENSTROM, J. *Am. J. Med.* 22: 758, 1957.
38. WATSON, C. J. PORPHYRIA In: *Advances in Internal Medicine*, Vol. 6, edited by W. Dock and I. Snapper. Chicago: Yr. Bk. Pub., 1954, p. 235.
39. WATSON, C. J. AND E. A. LARSON. *Physiol. Rev.* 27: 478, 1947.
40. WATSON, C. J. AND S. SCHWARTZ. *J. Clin. Invest.* 20: 440, 1941.
41. WIRTSCHAFTER, J. D., F. W. TURNER AND R. S. DOW. *Neurology* 10: 787, 1960.
42. WOODS, S. M., H. A. PETERS AND S. A. M. JOHNSON. *A.M.A. Arch. Dermat.* 77: 559, 1958.

DISCUSSION

Metal-binding by pyridoxal derivatives and possible relationships to tryptophan metabolism[1]

DAVID E. METZLER

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa

IN THE STUDIES REPORTED by Drs. Price and Peters, patients with certain metabolic disorders have been found to display an abnormal excretion pattern of tryptophan metabolites. Many of the same patients excreted excessive amounts of zinc and copper and were clinically benefited by treatment with complexing agents such as ethylenediaminetetraacetic acid (EDTA) and 2,3-dimercaptopropanol (BAL). A pronounced zinc and/or copper diuresis was observed during the

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early stages of the chelation therapy. Three tentative conclusions—or perhaps I should say hypotheses—have been suggested by one or both of these workers.

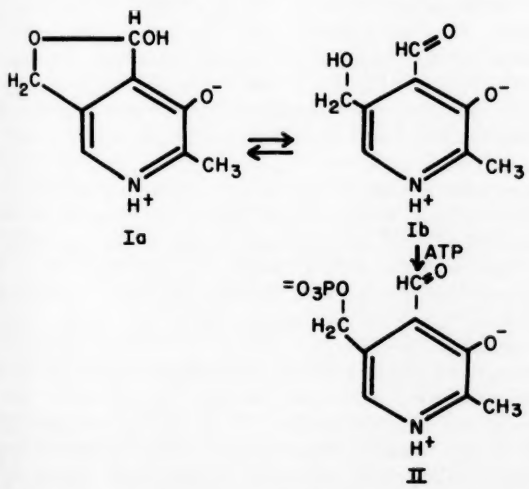
1) Metal toxicity may be an important aspect of these diseases.

2) The activities of two vitamin B_6-dependent enzymes, kynureninase and kynurenine transaminase are often decreased in patients with these diseases.

3) Since metal ions are known to form chelate complexes with imines (Schiff bases) of vitamin B_6 aldehyde (pyridoxal), it is possible that a metal ion imbalance is responsible for the observed effects upon the action of the vitamin B_6-dependent enzymes of tryptophan metabolism (23, 24).

The first two of these hypotheses seem to be completely consistent with the data presented. In both cases, it is to be expected that in time more direct evidence will be forthcoming. As Dr. Peters has suggested, metal balance studies on patients prior to, as well as during, the acute stages of these diseases and during the course of chelation therapy would be very revealing—although no doubt difficult to obtain. A direct demonstration of the postulated decrease in activity of the two enzymes kynureninase and kynurenine transaminase would also be of great interest.

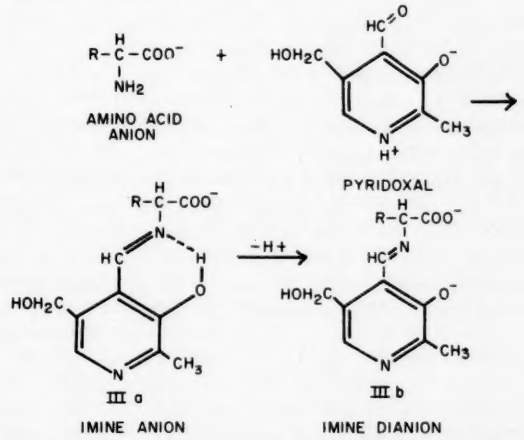
Most of my remarks today will concern the third suggestion. I will begin by describing some recently completed work from Iowa State University which gives a quantitative description of the formation of these metal chelates of imines derived from pyridoxal and amino acids (6).



Pyridoxal, one member of the vitamin B_6 family, exists in neutral solutions principally as the dipolar ionic cyclic hemiacetal, form Ia, in equilibrium with a small amount of the free aldehyde form, Ib (22). This aldehyde is believed to be a component of prime importance in the active site of a great variety of enzymes which act upon alpha amino acids (2, 26). Participation in these enzymic reactions depends upon a prior conversion of pyridoxal to its coenzyme form, pyridoxal phosphate (II). This conversion requires the action of a special enzyme, pyridoxal kinase, which has recently been partially purified from brain tissue by McCormick and Snell (19).

Unlike pyridoxal, pyridoxal phosphate cannot exist as a cyclic hemiacetal but remains as the free aldehyde form, and, in part, as a hydrate of the free aldehyde (22). For several technical reasons, we have used pyridoxal rather than pyridoxal phosphate in the studies described here.

The neutral hemiacetal form of pyridoxal (Ia) absorbs ultraviolet light strongly with the maximum absorption at a wave length of about 317 mμ (22). This light absorption affords a simple means of following the concentration of this substance in solution. In the presence of an alpha amino acid, a dramatic change in the light absorption of pyridoxal occurs with the appearance of a strong absorption band with a maximum at about 414 mμ (20). The absorption extends into the wave length region of visible light enough to impart a deep yellow color to the solutions. The 414 mμ absorption band is caused by the presence in the solution of an imine or Schiff base (IIIa) formed from pyridoxal and the amino acid. At pH values above 10, the imine anion (IIIa) loses the hydrogen bonded proton to form an imine dianion (IIIb) which absorbs light maximally at about 370 mμ (20).



If a salt of a suitable metal such as zinc is added to a solution of pyridoxal and an amino acid, the yellow color of the imine fades immediately; the solution develops a strong blue-green fluorescence and the light absorption maximum shifts to about 380 mμ. Since no such absorption band arises from zinc and pyridoxal alone or zinc and amino acid alone, this band must result from the formation of a metal complex of the imine (6).

Figure 1 shows the complete absorption spectrum of the zinc chelate of the imine derived from pyridoxal and the amino acid valine at several pH values. This spectrum could not be measured directly because we found it impossible to obtain complete conversion of pyridoxal to this chelate in solution. Some free pyridoxal

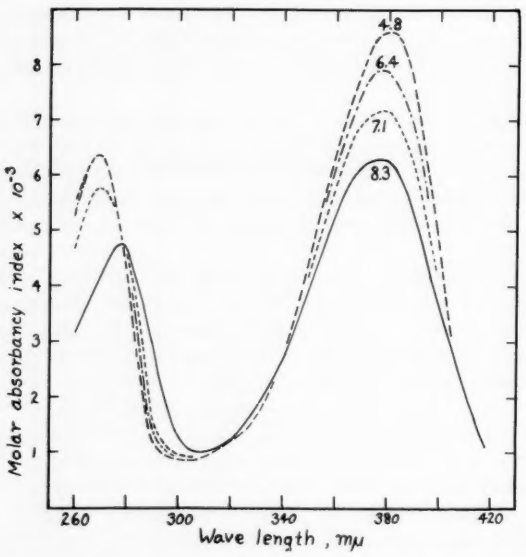
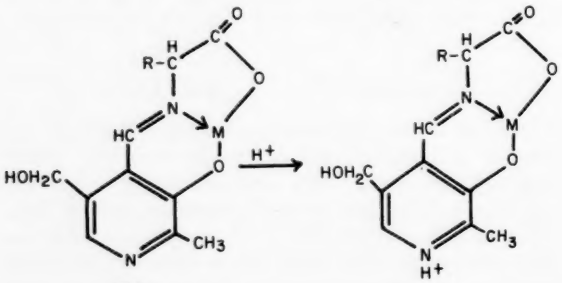


FIG. 1. Light absorption spectrum of the zinc chelate of the pyridoxal-valine imine at various pH values (indicated beside the curves).

and unchelated imine were always present. We have estimated the residual absorption of these forms and have subtracted it from the observed spectra. Figure 1 represents this difference, after conversion to a molar basis. A probable explanation of the variation of the absorption spectrum of the chelate with pH is that at high pH the metal complex has structure IVa in which the metal ion replaces the dissociable hydrogen atom of the imine IIIa. At lower pH values this chelate accepts a proton on the pyridine nitrogen to give Structure IVb with an altered absorption spectrum and lowered fluorescence. From the change in the absorption spectrum of the zinc chelate with pH, we can estimate the dissociation constant, as an acid, of the protonated form of the chelate. Unfortunately, at high pH values the



variation of the absorbancy at the peak wave lengths with pH is not quantitatively consistent with this simple theory (6). However, we are inclined to attribute these deviations to a number of experimental errors. In favor of this view is the observation that the plot of the ratio of the absorbancy at 270 mμ to that at 380 mμ against pH is a titration curve of the theoretically

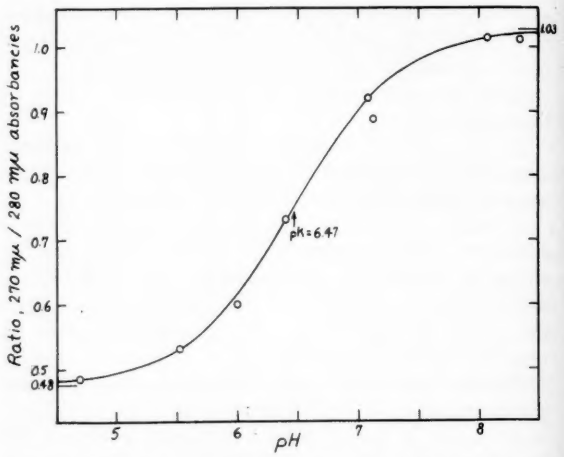


FIG. 2. Ratio of absorbancies at 270 mμ and 280 mμ against pH for the zinc-pyridoxal-valine chelate. The solid line is a theoretical curve for pK = 6.47 and limiting ratios of 0.48 and 1.03.

expected shape (Fig. 2). The logarithmic dissociation constant, pKa, is 6.5.

These results are very similar to those obtained by Christensen (4) and confirmed by us (6) for the copper-pyridoxal-valine system. In this case a pKa value of 5.6 is observed for the metal chelate. Very similar results have also been obtained for a variety of other amino acids and for several other metals. Of these nickel has been studied in some detail and Co, Fe, V, Mn and Al have been studied but slightly (6).

An important problem in interpretation of our results arises from uncertainty about the composition of the imine chelates which are recognizable spectrophotometrically. Although we assumed in writing structures IVa and IVb that pyridoxal, amino acid and metal combine in a 1:1:1 molar ratio, we could not be certain of this without further experimentation. Indeed, there were some reasons for suspecting a different composition. For example, Eichhorn and Dawes (7) showed by the method of continuous variation that in the pyridoxal-alanine-nickel system a chelate with a 2:2:1 ratio of components was present. Similar results for aluminum chelates have been reported by Fassela *et al.* (8, 9). These complexes presumably contain two imine molecules bound to a single metal ion. Christensen (3) has isolated solid chelates of pyridoxal-valine imines with zinc, nickel and manganese with a 2:2:1 ratio of components. It is also possible that chelates occur in which one imine ligand and one other amino acid ligand are bound to the metal ion to give a 1:2:1 ratio of components. The observations by Longenecker and Snell (15, 27) of copper and pyridoxal-catalyzed transamination of L-amino acids with partial retention of configuration strongly suggest that one or another of these higher chelate types may function in nonenzymic catalysis by pyridoxal and metal salts.

In addition to learning more definitely the composition of the chelates in solution, we were anxious to obtain

quantitative information about the stability of the complexes. The problems of establishing the composition and of evaluating the stability constants actually are closely related. We anticipated that in the most dilute solutions a 1:1:1 type of chelate would doubtless predominate. If its stability constant could be evaluated, we could subsequently measure the apparent stability constant under a variety of conditions involving drastic alterations in concentrations of the various components. We expected that any dependence of the apparent stability constant on the valine or pyridoxal concentrations might be indicative of formation of chelates of the 2:2:1 or 1:2:1 types. The pH dependence of the apparent constant would also give us an independent evaluation of any hydrogen ion binding equilibria in the chelates.

Our procedure for evaluation of the apparent constant K was essentially as follows: Since

$$K = [\text{Imine chelate, all forms}]/[\text{Imine dianion}][M^{++}]$$

it was necessary to evaluate the concentrations of the imine chelate, free imine dianion and free metal ion in a solution at equilibrium. A series of such solutions were prepared, all of ionic strength of 0.1 and containing substantial amounts of both free pyridoxal or free imine and of the metal chelates of the imine. Knowing the absorption spectra of both the chelate and free pyridoxal, we could estimate the amounts of each in any solution through the use of light absorption measurements, principally at 315 and 380 mμ. Absorption at 420 mμ was also measured and used in estimating the amount of free imine present (6). The free amino acid concentration was usually in large excess and was readily estimated. From the free amino acid concentration, the spectrophotometrically estimated free pyridoxal concentration and known equilibrium constants (20) it was a simple matter to compute the concentration of the dianion of the free imine.

Evaluation of the free metal ion concentration was a little more involved. Metal ions can be expected to complex with things other than the imine including free amino acids and free pyridoxal. Study by both titration and spectrophotometry indicated that the complexes of metals with pyridoxal were very weak and could safely be ignored in our calculations (6). On the other hand, the 1:1 and 2:1 complexes of amino acids with metal ions are very strong. Thus, in any reaction mixture we will find the metal ions which are not bound to the imine distributed among several forms including free metal ions, the 1:1 amino acid chelate and the 2:1 amino acid chelate. In order to evaluate the free metal ion concentration in a solution, it was necessary to evaluate this distribution which involved knowing the formation constants of the two types of amino acid-metal chelates. Because of the great variation among literature values, we found it necessary to re-evaluate these constants under our experimental conditions. With this information, it was possible to calculate the free metal ion concentration. From the free metal, the free imine

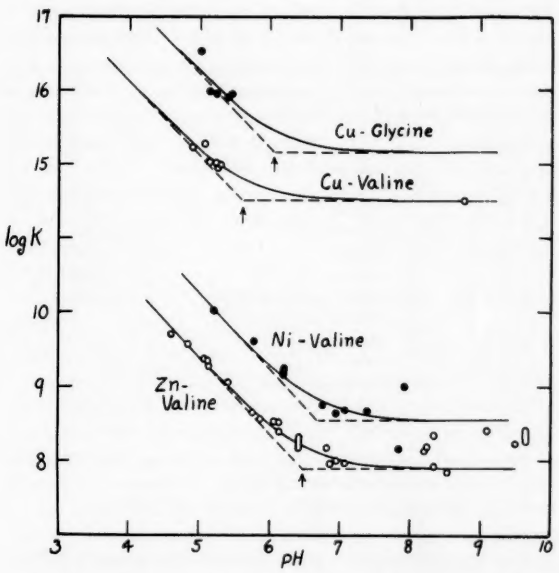


FIG. 3. Apparent formation constants K for pyridoxal imine chelates. The solid lines are theoretical curves based on the assumption that the chelate accepts a single proton with the pK indicated by the vertical arrows. These pK values were independently obtained except in the case of the nickel-valine system.

dianion and the spectrophotometrically determined imine chelate concentrations it was possible to evaluate the desired formation constant. The whole procedure is rather involved, making occasional use of successive approximation methods, and is described in more detail elsewhere (6).

Since at low pH the imine chelate is protonated, the formation constant obtained is pH dependent, becoming pH independent at higher pH. In Figure 3 are shown the experimentally obtained values of the formation constants obtained for three different metals complexed with the pyridoxal imines derived from two different amino acids. The data cover solutions in which the total concentrations and ratios of the three components were varied widely (6). The solid lines are curves of the theoretically expected shape using the independently determined pKa values of 6.5, 5.6 and 6.1, respectively, for the protonated forms of the zinc-valine, copper-valine and copper-glycine imine chelates. The maximum curvature of the theoretical lines fall at these pK values.

The generally good fit of the data to the theoretical curves which were derived on the basis of a chelate of 1:1:1 composition demonstrates clearly that it is these 1:1:1 chelates which predominate in the solutions studied. The more scattered data at high pH reflect the increasing difficulty of accurate calculation at high pH. It is possible that some of the deviations from the theoretical line result from the presence of other forms of chelate, but we were unable to find a satisfactory quantitative explanation of these data. The results for the copper-pyridoxal-valine chelate are quantitatively

TABLE 1. *Formation Constants, K_c of Metal Complexes of Imines of Pyridoxal and Amino Acids and of Other Ligands*‡

Constants are for the reaction of the dianion of the imine with the metal ion to form a complex containing a 1:1:1 ratio of metal, pyridoxal and amino acid.

Metal Ion	Ligand	Log K_c
	Imines derived from	
H^+	Valine	10.5
Cu^{++}	Valine	14.5
	Glycine	15.0
Ni^{++}	Valine	10.8
Zn^{++}	Valine	7.94
	Other amino acids*	7.4–8.1
	Isobutylamine	<5.2
Mn^{++}	Valine	5.0
Mg^{++}	Valine	<3.5
Zn^{++}	Pyridoxamine	5.7†
	Valine (free anion)	4.5
Ni^{++}	Pyridoxal	*ca.* 2.0

* Glycine, alanine, threonine, leucine, isoleucine, glutamic acid. † Gustafsoe and Martell (12). ‡ (Davis, L., F. Roddy and D. E. Metzler. *J. Am. Chem. Soc.* 83: 127, 1961).

in excellent agreement with the results reported earlier by Christensen (4).

Although no evidence of 2:2:1 chelates was obtained in this study, it is probable that they do form in more concentrated solutions with higher ratios of free imine to metal concentrations. As mentioned earlier, a solid light yellow valine-pyridoxal-zinc chelate can be isolated and is found to contain a 2:2:1 ratio of components. It is interesting that this solid complex has such a radically different composition from the form predominating in solution. When the solid chelate is dissolved in water, it breaks down rapidly to give the expected mixture of pyridoxal and the 1:1:1 chelate. In methanol, the 2:2:1 chelate is more stable and has an absorption spectrum very similar to that of the 1:1:1 chelate. Apparently, no radically different bonding exists in the two chelates. This observation is of interest in connection with the suggestions of Christensen (3) about the metal bonding in these complexes.

The formation constant data in Figure 3 can all be summarized by means of a pH independent constant for formation of the unprotonated chelate. The values of these pH independent formation constants are given in Table 1 along with estimated values for some other metals and amino acids. Data on several other chelates are included for comparison purposes. Several things are evident from this table. The formation constants of the chelates are relatively high, higher, for example, than these of either free pyridoxal or amino acids. The imine of pyridoxal with isobutylamine, however, does not chelate to a measurable extent. The importance of the tridentate nature of the pyridoxal-amino acid imines in the formation of these strong chelates is thus evident. The order of affinity of the various metals for the imines is the one normally encountered in chelate chemistry (16).

An unexpected finding was that the zinc-pyridoxal-amino acid chelates are remarkably unstable with respect to light. In one experiment irradiation of a chelate-containing solution by placing it in a 1 cm quartz curvette inside a north window of the laboratory resulted in a 50 per cent decrease in the 377 mμ absorption in 2.5 hours. This irradiation appears to convert the chelate almost quantitatively to an unstable compound of unknown structure with a maximum absorption at 330 mμ. Although we cannot immediately suggest any biological role for this phenomenon, some biological significance may well exist for such an extremely photosensitive compound. Many other metals including copper and nickel form much less fluorescent chelates which do not rapidly undergo this photochemical reaction.

In biological systems we are often more interested in pyridoxal phosphate than in pyridoxal itself. Strong chelate complexes are formed with imines of pyridoxal phosphate (18) but our description of these systems is less complete. Doubtless because of the lack of internal hemiacetal formation in pyridoxal phosphate, it tends to form imines more readily than does pyridoxal. Basing estimates in part upon an estimate of the amount of free aldehyde present in pyridoxal (22), and in part upon data of Matsuo on the stability of imines of pyridoxal phosphate (18), we predict imine chelate formation to be about 50–100 times as strong with pyridoxal phosphate as we have observed with pyridoxal. The presence of the phosphate may enhance the stability even more.

Biochemists have often considered the possibility that metals might be components of vitamin B_6-dependent enzymes. In the present context I thought it also might be advisable to ask whether it is even possible that free pyridoxal phosphate, amino acids and metals complex nonenzymically within body tissues. This question cannot really be answered because as far as I know we can only guess at the concentrations of the free reactants in tissues. However, if concentrations of free pyridoxal phosphate, amino acid and zinc reached levels of 10^{-6}, 10^{-3} and 10^{-5} M, respectively, we would find about as much pyridoxal phosphate existing as imine chelates as is free. Thus, such chelation could conceivably be of importance in competing with enzyme sites for pyridoxal phosphate. Such competition could occur with a much lower concentration of free copper. Perhaps pyridoxal phosphate could even be destroyed or converted to inhibitory compounds in a light-catalyzed reaction under the influence of zinc. I am not suggesting these as really likely possibilities, but perhaps a discussant is expected to be a little imaginative and to suggest some ideas that may be far-fetched but worth examining.

The discovery that metals catalyze nonenzymatic reactions of pyridoxal with amino acids suggested immediately (1, 21) that metals might participate in the action of pyridoxal phosphate-dependent enzymes. As pointed out by Snell in these proceedings, this idea is not born out very well by experiment. Of the few enzymes of this group which have been purified highly,

none has been demonstrated to require a metal for activity. In the present discussion the enzymes kynureninase and kynurenine transaminase are of particular interest. Unfortunately, in neither case are highly purified preparations available for study. Activation of *Neurospora* kynureninase by manganous and calcium ions has been reported by Saran (25). Jakoby and Bonner (14) have reported activation by magnesium ion, but Saran reports inhibition by magnesium, cobalt, zinc and various other metal salts at 10^{-5} M concentrations. Despite these apparent metal activation effects, Saran was unable to demonstrate removal of an essential ion by dialysis against EDTA solutions. *Neurospora* kynurenine transaminase has also been reported to be partially activated by magnesium (13).

Similar metal activation effects have been reported for several impure pyridoxal phosphate-dependent enzymes (2). Such reports of partial activation of enzymes in impure preparations must be interpreted with caution. Many unsuspected reactions can occur. For example, a metal might act as a protective agent by complexing with an inhibitory compound, or it might activate another enzyme whose activity would benefit the system under study. Zinc and other divalent ions, for example, activate pyridoxal kinase, the enzyme responsible for formation of pyridoxal phosphate from free pyridoxal (19); and it is conceivable that under some experimental conditions this could lead to apparent metal activation of a pyridoxal phosphate-requiring enzyme. Possibly inhibition of pyridoxal kinase through zinc deficiency could be one cause of the apparent vitamin B_6 deficiency symptoms observed during EDTA treatment which Dr. Price has mentioned.

Even when a clear-cut metal requirement is demonstrated, it is important to realize that this does not necessarily mean that the metal is bound to a pyridoxal imine group at the enzyme active site. In the case of tryptophanase, another pyridoxal phosphate-dependent enzyme of bacteria, a strong activation by the univalent ions K^+, NH_4^+ and Rb^+ has been reported by Happold and Beechey (13). Yet these ions are catalytically inactive in the nonenzymic model reactions of pyridoxal with amino acids and they complex very weakly, if at all, with pyridoxal imines. An interaction at some other site on the enzyme is likely (13).

Turning from activation to inhibition, we find that various pyridoxal-containing enzymes in addition to kynureninase are inhibited by metal ions. Inhibition of enzymes by heavy metals is a very common phenomenon and is certainly not limited to pyridoxal-dependent enzymes. Again, it is not safe to assume that an inhibitory metal is bound to the co-enzyme, though that is a very definite possibility. The fact that in purified pyridoxal phosphate-dependent enzymes the pyridoxal appears to be combined in the resting enzyme with an ϵ-amino group of lysine as an imine (10, 11) shows that these enzymes possess a site which may be susceptible to metal ion inhibition. However, it should be noted that this type of imine with lysine, like the one derived from isobutylamine may have a rather weak affinity for metals (Table 1).

Unfortunately, little pertinent information is available on mammalian kynureninase and kynurenine transaminase. The first of these enzymes has been studied by Dalgliesh *et al.* (5) and by Wiss and Weber (28). The latter workers could demonstrate no metal activation effects. Kynurenine transaminase has been studied by Mason (17), but no metal effects have been reported. Certainly, further studies of these two enzymes should be encouraged.

Finally, as a chemist, is there anything that I can suggest regarding the complex biological situations being considered by Drs. Price and Peters in their clinical studies? The first thing for any of us to recognize, I believe, is the tremendous complexity of any situation in which we are considering the distribution of metal ions among various complex biochemicals including proteins. In our own studies, we were somewhat amused at the complexities, not all of which I have mentioned today, which were encountered in this very simple artificial situation. With so many reactions competing for the metal ion, we found that even after we thought that we understood the picture rather well, we had a hard time predicting just what would happen in a given solution without lengthy calculations. In any biological situation with multiple compartments and transport rates as well as equilibrium constants to be considered, we have an enormously greater complexity. Exact explanations of metal toxicity effects are likely to be very elaborate. The effects on tryptophan metabolism observed by Dr. Price might be either a direct or indirect result of elevated metal levels. Decreased enzyme activity might result directly from metal ion inhibition or indirectly through metal inhibition of enzyme synthesis. It is even possible that it is not metal inhibition which is observed in these patients, but a faulty distribution of certain metals in the body. We are not dealing with an equilibrium situation but with a dynamic steady state, and it is possible to imagine situations in which a chelating agent like EDTA could remedy a metal deficiency at one location by facilitating redistribution of the metal in the body.

In order to try to understand these diseases, what further things would a biochemist like to know? First of all, he would probably want to know whether or not the total concentration of metals in tissues is abnormally high. Extensive analytical data on tissues including blood would be very valuable. If an abnormality in the total amount of a metal is found, the distribution of the metal among various bound forms can then be studied and clues to an understanding of the disease may be revealed. Thus, studies of serum copper have revealed a decreased level of the specific protein ceruloplasmin in Wilson's disease as noted by Dr. Scheinberg in this symposium. In addition to tissue analyses, metal balance data on patients would be very valuable, as Dr. Peters has suggested.

Another kind of information also would be of great interest. In contrast to the total metal concentration in a tissue, how does the concentration, or thermodynamic activity, of free metal ion vary in these disease conditions? Is it raised to abnormally high levels continuously or intermittantly? This may be a little harder to learn; but, if we had such information, it would be possible to predict, from studies of purified enzymes, which enzymes would be especially susceptible to metal poisoning under particular conditions of metal ion imbalance. We could then attempt to see whether or not clinical symptoms could be explained by these enzyme inhibitions.

It is to be hoped that more pertinent chemical data with respect to these diseases will be obtained in the future. In the meantime we can be grateful for the progress which is being made in treating these conditions by chemical means.

REFERENCES

1. Baddiley, J. *Nature, London* 170: 711, 1952.
2. Braunstein, A. E. In: *The Enzymes* (2nd ed.), Vol. 2, edited by P. D. Boyer, H. Lardy and K. Myrbäck. New York: Acad. Press, 1960, p. 113.
3. Christensen, H. N. *J. Am. Chem. Soc.* 79: 4073, 1957.
4. Christensen, H. N. *J. Am. Chem. Soc.* 80: 2305, 1958.
5. Dalgliesh, C. E., W. E. Knox and A. Neuberger. *Nature, London* 168: 20, 1951.
6. Davis, L., F. Roddy and D. E. Metzler. *J. Am. Chem. Soc.* 83: 127, 1961.
7. Eichhorn, G. L. and J. W. Dawes. *J. Am. Chem. Soc.* 76: 5663, 1954.
8. Fasella, P., H. Lis, C. Baglioni and N. Siliprandi. *J. Inorg. & Nuclear Chem.* 8: 620, 1958.
9. Fasella, P., H. Lis, N. Siliprandi and C. Baglioni. *Biochim. et biophys. acta* 23: 417, 1957.
10. Fischer, E. H., A. B. Kent, E. R. Snyder and E. G. Krebs. *J. Am. Chem. Soc.* 80: 2906, 1958.
11. Fischer, E. H. and E. G. Krebs. *Abstracts of Papers: 136 Meeting Am. Chem. Soc.*, Atlantic City, N. J., 1959, p. 24c.
12. Gustafson, R. L. and A. E. Martell. *Arch. Biochem. Biophys* 68: 485, 1957.
13. Happold, F. C. and R. B. Beechey. In: *Metals and Enzyme Activity* (Biochemical Society Symposium No. 15), edited by E. M. Crook. London, Cambridge, 1958, p. 52.
14. Jakoby, W. B. and D. M. Bonner. *J. Biol. Chem.* 205: 699, 1953.
15. Longenecker, J. B. and E. E. Snell. *Proc. Nat. Acad. Sci.* 42: 221, 1956.
16. Martell, A. E. and M. Calvin. *Chemistry of the Metal Chelate Compounds*. New York: Prentice-Hall, Inc., 1952, p. 181.
17. Mason, M. *J. Biol. Chem.* 234: 2770, 1959.
18. Matsuo, Y. *J. Am. Chem. Soc.* 79: 2011, 1957.
19. McCormick, D. B. and E. E. Snell. *Proc. Nat. Acad. Sci.* 45: 1371, 1959.
20. Metzler, D. E. *J. Am. Chem. Soc.* 79: 485, 1957.
21. Metzler, D. E. and E. E. Snell. *J. Biol. Chem.* 198: 353, 1952.
22. Metzler, D. E. and E. E. Snell. *J. Am. Chem. Soc.* 77: 2431, 1955.
23. Price, J. M. and R. R. Brown. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 179.
24. Price, J. M., R. R. Brown and H. A. Peters. *Neurology* 9: 456, 1959.
25. Saran, A. *Biochem. J.* 70: 182, 1958.
26. Snell, E. E. *Vitamins and Hormones* 16: 77, 1958.
27. Snell, E. E. and W. T. Jenkins. *J. Cell. & Comp. Physiol.* 54 (Supplement 1): 161, 1959.
28. Wiss, O. and F. Weber. *Hoppe-Seyler's Ztscher. physiol. Chem.* 304: 232, 1956.

GENERAL DISCUSSION

Dr. Cotzias: With the danger of being accused of establishing a mutual admiration society with Dr. Peters I wish to congratulate him on this study. Ordinarily, neurologists are concerned more with diagnosis of disease than treatment but Dr. Peters is an exception. I would like to ask Dr. Peters if he has any analyses of tissues, particularly of brain and peripheral tissues, in these studies.

Dr. Peters: I am sorry, but we do not have much data on tissue analyses. We have been handicapped by having very few deaths in our series. I think that Dr. Butt might be able to comment on his analyses of tissues for I know that in the field of alcoholism he has extremely elaborate information as to the liver and brain content of some of the cations under consideration.

Dr. Edward M. Butt (University of Southern California School of Medicine, Los Angeles): We have completed recently the examination by emission spectroscopy of tissue from one hundred cases of alcoholism with hepatic cirrhosis. The examination was limited to thirteen metals in thirteen tissues. The cases were arbitrarily divided into groups according to the level of hepatic iron.

About 60 per cent of the cases had less than the normal amounts of hepatic iron. Not only was hepatic iron found to be low but other organs had less than the usual amounts of iron. Associated with reduced iron, there was a decrease in the levels of zinc, molybdenum, and cobalt and an increase in copper and silver. Reduced zinc levels have been reported before by Vallee (Vallee, B. L., W. E. C. Wacker, A. F. Bartholomay and F. L. Hoch. *Ann. Int. Med.* 50: 1077, 1959). Molybdenum in most cases disappears within the limits of the method and xanthine oxidase activity is reduced (Waterlow, J. C. *Fed. Proc.* 18: 1143, 1959). Aluminum levels are about normal.

The organs of the remaining 40 per cent of the cases of alcoholism with cirrhosis of the liver store iron, except for the brain. The hepatic iron in these cases varies from small increments to enormous quantities noted in idiopathic hemochromatosis. The hepatic metal pattern except for quantitative

variation is essentially the same as in hemochromatosis, consisting of increases in the levels of lead, molybdenum, and calcium with a decrease in aluminum. The increases in the levels of lead may reach or surpass the values in lead poisoning. These patients do not have symptoms or signs of lead poisoning and it is postulated that a natural chelating agent is present which most likely is hemosiderin. This tenfold or more increase in lead is not found only in alcoholics with hepatic iron storage but is noted in Bantu siderosis and all other forms of excessive hepatic iron storage, such as Mediterranean anemia, sickle cell anemia and megaloblastic anemias. Zinc levels which are low in the reduced iron group are noted to return to about normal levels in the iron storage group. This increase in zinc levels may be due to the fact that zinc has become a storage metal and has little physiological activity similar to the situation in cattle with normal hepatic copper levels manifesting copper deficiency due to an excess intake of molybdenum (McElroy, W. D. and B. Glass, (editors). *Copper Metabolism.* Baltimore: Johns Hopkins Press, 1950).

We have had four cases of acute porphyria. The organ metal patterns are within normal limits except for a slight reduction in the levels of zinc and calcium. Lately, we have been determining serum zinc levels in cases of delirium tremens and hepatic coma in alcoholics. In both diseases, we have found the zinc levels to be quite low, and the administration of zinc as zinc sulfate by mouth has resulted in a return of the serum zinc to normal levels. The clinician responsible for this treatment has stated that the patients receiving zinc appear to benefit from this additional treatment.

In alcoholics with cirrhosis of the liver, there are two distinct groups, one in which most of the trace metal levels of tissues are low, particularly iron, and a second group in which iron is stored. The organs involved in the storage of iron, as well as the hepatic trace metal pattern, are the same as noted in idiopathic hemochromatosis.

An interesting situation arises in attempting to get serum zinc control values. Most groups of individuals imbibe moderate amounts of alcohol or have dietary variations that in all probability affect their zinc levels. At the present time we are receiving serum from members of a religious group who are opposed to the use of alcohol and eat a normal diet containing meat. In a variety of individuals we have found the zinc levels to vary from 20 to 200 micrograms per 100 cc of serum.

Dr. Kehoe: Certain implied questions may be raised with respect to the evidence that the zinc metabolism, in particular, but the copper metabolism, as well, is as badly deranged as it may appear to be. I've no right to question this, but, seriously, I do raise the point that one cannot consider the concentration of these metals in the urine as evidence of abnormality. If the concentration can be calculated on the basis of a normal water intake as being within normal limits, you are dealing strictly with normal metabolism. Any one of these metals can be increased enormously in its concentration in the urine by simple deprivation of water. In consequence, the urinary concentration alone may be grossly misleading so far as metabolism is concerned. In this situation, too, I should think one would have to have evidence indicating the levels of intake per day in order to be able to interpret the output in terms of being within the realm of normality or abnormality. I call attention, also, to a matter that was suggested by the last discussion, namely the depletion of metals in the alcoholic. The alcoholic notoriously starves himself and drinks his meals. This is an ideal situation for the depletion of the metals of the body. The longer the alcoholic has been on this alcoholic diet the more will the metals of the body be depleted. I'm not surprised in these circumstances to find that there is very little metal left, for example, in the liver.

Dr. Peters: I would like to say, first of all, that the figures given in these cases included some patients with oliguria, but many also had a normal urinary output. We did note that as normal output was established by forcing fluids in oliguric patients the concentration of zinc and copper remained in the pathological range. I agree completely with the necessity for balance studies which we are attempting to set up now. Dr. Butt has pointed out that the zinc concentration is reduced in the alcoholic liver. Dr. Vallee has suggested that this decreased concentration could be due to the increase in connective tissue in the liver, which ordinarily does not store zinc.

We have taken selected alcoholics (some of whom may have had Laennec's cirrhosis) who were suffering clinically with alcoholic neuropathy, Wernicke's encephalopathy manifesting extraocular muscle paralysis or delirium tremens and administered EDTA or, in a few cases, BAL. This has brought about clinical improvement, in some instances, despite the fact that we actually were greatly augmenting the zinc excretion with these agents. We cannot dismiss the possibility that these chelating agents might bring about a redistribution of these cations within the body which is more compatible with normal metabolism. Perhaps we would be better off if we could give chelating agents that would not be excreted, but the marked augmentation of zinc and copper excretion after EDTA or BAL administration in the porphyric and in some of these other conditions does not appear to be incompatible with the individual's improving, sometimes very rapidly. Then, too, perhaps the chelate merely removes the heavy metals that concievably block certain metalloenzymes in the body.

I was interested in the material Dr. Metzler mentioned as to the effect of light on certain enzyme systems. We have mentioned that some of the acute porphyrics and almost all of the chronic porphyrics are aggravated by exposure to ultraviolet light. One acute porphyric developed her first symptoms after falling asleep in the sun and getting a severe sunburn. Many of these patients do better if they avoid sunbathing. It has been pointed out also that ultraviolet light will precipitate lead colic not infrequently. We have pointed out that zinc and copper urinary excretion increases in patients intoxicated with lead, arsenic or mercury.

Dr. Price: It is of interest that several of the intermediary tryptophan metabolites should be very good chelating agents. For example, 2-carboxyquinoline derivatives are good metal-binding agents and there are at least five of these produced from tryptophan (cf. Fig. 1, my manuscript). One of these, namely kynurenic acid, is known to circulate in the enterohepatic circulation. It is possible that kynurenic acid is absorbed from the intestine as a chelate. Kynurenic acid may be excreted in the bile as a complex with iron, copper or manganese, for example, since these metals also are known to be excreted in the bile. There is an increase in the production of these tryptophan metabolites in pyridoxine deficiency and Gubler, Cartwright and Wintrobe showed (Gubler, C. J., G. E. Cartwright and M. W. Wintrobe. *J. Biol. Chem.* 178: 989, 1949) that the iron and copper content per 100 gm body weight of rat is increased in pyridoxine deficiency. It appears possible, therefore, that some of the intermediary tryptophan metabolites may assist in the transport of metal ions into or out of the body, or both.

Dr. Philips: Can you tell us if the patient with porphyria progresses downward steadily once his disease is recognizable or are there acute exacerbations followed spontaneously by episodes of apparent normality? If porphyria is a disease of

varying intensity at different times in the same individual, how do you distinguish between a spontaneous remission and a beneficial therapeutic effect?

Dr. Peters: I want to thank Dr. Philips for his question, which is extremely appropriate in view of the remissions and exacerbations that notoriously occur in some of these cases of acute porphyria. For this reason, cases that were not extremely ill were treated frequently by alternative measures, such as the use of phenothiazine derivatives. Naturally, in all instances, we avoided exposure to barbiturates and sulfa drugs once the diagnosis had been made. Frequently, these alternative measures, however, were not adequate either to bring about complete clinical remission or to prevent additional exacerbations. We then would introduce a chelating agent, such as EDTA, and would compare the clinical response. I would like to emphasize that our follow up periods have been over a matter of years in some instances. On the other hand, there are cases of porphyria, for example those precipitated into an acute crisis by a laparotomy under pentothal anaesthesia, who begin to convulse, become psychotic, and who develop severe peripheral neuropathy, anuria, oliguria, etc. We have treated some of these people during these periods of rapid deterioration and have noted, at times, striking improvement. Since many of these patients had been treated ineffectively with steroids prior to our chelating efforts, we were comparing also the effectiveness of one drug against another. In one of our previous papers we reported on one man who deteriorated while receiving BAL during such a severe crisis, this man was saved, I believe, only by the addition of ACTH. In retrospect, this fellow had done a great deal of painting and his acute episode was precipitated by a period of painting. He had a borderline lead level in his blood and I think that we may well have enhanced this lead toxicity by the use of BAL. Since that time we have always used EDTA preferentially when the lead level was raised even slightly, or when there was a known exposure to lead in the history.

Some of our patients had been hospitalized for months with symptoms of neuropathy or psychosis that failed to respond to other measures. We have taken some of these patients, given them EDTA, frequently following this with a course of BAL, and have been able to demonstrate almost immediate response as far as their neuropathy and psychosis is concerned. It should be noted that the neuropathy, when it develops, consists of a demyelinization and probably a variable destruction of the nerve fiber. This means that for complete recovery from neuropathy we sometimes have had to wait for as long as a year for the nerves to grow back and become functional. We do have several movies that show some of these severely involved patients before and after treatment. Psychotic symptoms, when they exist and are relatively acute, may be terminated by the administration of chelating agents. Epileptic phenomenon when these occur in the porphyric patients are often made worse by Dilantin (one of the most commonly used anticonvulsants). When multiple seizures are occurring in the porphyric, we have tried to avoid anticonvulsant drugs with the exception, at times, of paraldehyde or Avertin but have noted in some instances rather immediate control of seizure activity following the administration of these chelating agents.

Lastly, I would like to mention certain chronic and mixed porphyric patients who show light sensitive skin lesions that may be extremely disfiguring and who may, under certain circumstances, develop abdominal colic or other neurological symptoms. If some of these people go out in the sun their faces swell, they develop bulli and their skin breaks down and becomes infected. They are almost always made worse if they drink alcohol and they are made worse by chloraquine and by sulfa and barbiturate drugs. One of these patients has been followed for about five years and following a course of intravenous EDTA and subsequent administration of oral EDTA, which he continued until two years ago, there has been complete recovery from his skin lesions. He is sweating again through the skin and he is able to go out in the sun, ride a motorscooter and hunt without precipitating symptoms. In addition to this, he drinks some beer and, although he continues to put out some abnormal porphyrins, he is virtually asymptomatic clinically at this point. This would not be the usual course of this disorder.

In conclusion, I should like to suggest that only through multiple trials of these agents with careful controls by multiple observers can we ever be completely certain as to the efficacy of chelation therapy in these porphyric patients. I feel that the results of such therapeutic trials should be reported, whether negative or positive, because above all, we don't want to be fooled.

Chelation therapy in circulatory and sclerosing diseases

A. J. BOYLE, N. E. CLARKE, R. E. MOSHER AND D. S. McCANN

Department of Chemistry, Wayne State University, and Research Division, Providence Hospital, Detroit, Michigan

Interest at Wayne State University in ethylenediaminetetraacetic acid (EDTA) began in 1947, at which time it was employed as an aid in analytical chemistry. Subsequently, it was used for decalcification of tissue preparations and eventually became of interest in animal experimentation. This work (2, 19) stimulated our interest in the compound for the study and treatment of circulatory and connective tissue diseases.

I. CIRCULATORY DISEASE

A. Valvular Heart Disease

The first patient, E. C., (Myers, G. B., L. T. Iseri, and A. J. Boyle, unpublished observations), in our experimental series was a male, 54 years of age with a calcified mitral valve. He was admitted to the City of Detroit Receiving Hospital in January, 1953. He twice had undergone cardiac catheterization shortly before the present admission and a commissurotomy was being considered. At the time of admission he was in severe cardiac decompensation. During the first month of hospitalization, all forms of therapy failed to alleviate his dyspnea, orthopnea and edema. It was decided at this time to test the effect on this condition of Na_2EDTA administered intravenously. Accordingly, the patient was given 5 gm of Na_2EDTA in 500 cc of 5 per cent glucose five days a week for four weeks or a total of 100 gm of the compound. Intravenous infusion time averaged approximately 1.5 hours.

At the end of the first six days, dyspnea, orthopnea and edema were improved markedly and after the eighteenth intravenous infusion patient E. C. walked up four flights of stairs with only a modicum of discomfort. He experienced a marked diuresis after the first six days of treatment and for several days thereafter. Following treatment the patient was catheterized again. The mean pulmonary arterial pressure had dropped approximately one-half. The pulmonary capillary pressure also was lowered, but the change was barely sufficient to be considered outside the limits of error inherent in the method. The cardiac output remained constant; therefore, the efficiency of cardiac work improved since pulmonary resistance fell remarkably. Following cardiac catheterization the patient developed what appeared to be a pulmonary infarct from which he never recovered fully. Another series of Na_2EDTA infusions in August of that year was of no benefit and the patient expired on September 28. An autopsy was performed. The mitral valve of the patient was grossly ulcerated and exhibited severe calcification. Particulate matter from the stony mass was examined petrographically and found to be birefringent. This would seem to indicate that the build-up of concentrations was not continuous, but was, in fact, intermittent. Surface granules from the lesion were rounded and friable. One large atherosclerotic plaque was demonstrated on the intimal surface of the aorta in the thoracic portion. More than half of the plaque was macular with a large excavation in the remainder. Both areas presented smooth surfaces. Figure 1 shows the plaqued area enlarged. The question of whether this plaque was being reversed or not remains unanswered.

Clinical manifestations of emboli to mesentery and lung were not verified by autopsy. A summary of findings are given below:

Cardiovascular disease, rheumatic and arteriosclerotic; Valvulitis, mitral, calcific, severe with stenosis and insufficiency; Fibrosis, myocardium; Hyperemia, acute passive, liver, kidneys; Infarction, old, kidney, right; Nephrosclerosis, arterial, arteriolar, severe; Bronchiectasis, left lower lobe; Emphysema, senile; Atrophy, pancreas; Glial nodules, brain and mild inflammation peripheral nerves.

Despite this unfortunate outcome, the patient's response had been so dramatic that another patient, W. O., male, 34 years of age, with inoperable rheumatic aortic valvular disease was studied. It was recognized that the salutary effect achieved with patient E. C. was not due to the direct action of Na_2EDTA on the mitral valve. Several ideas were entertained as to the mechanism and they will be discussed briefly later on. A calcium and magnesium balance study was thought desirable and undertaken. The experimental schedule consisted of ten periods of four days each during which time the patient was given a low calcium diet.

Figure 2 illustrates graphically results of the balance study on W.O. with respect to loss and retention of these elements. During periods 1, 4, 7, and 10 no Na_2EDTA was given. In the remaining periods the usual 5 grams of

FIG. 1. Aortic atherosclerotic plaque from patient E. C.

Na_2EDTA in glucose was administered daily. Total intake and output of calcium and magnesium for each period were determined. As might be expected, during Na_2EDTA treatment the bulk of calcium lost was *via* urine. During periods free of Na_2EDTA, the calcium loss was largely by way of the feces. In the case of magnesium, greater losses occurred in the stool except for periods 7 and 9. During this study, patient W. O. had a total net loss of 27.0 gm of calcium and 4.22 gm of magnesium. The patient received 120 gm of Na_2EDTA which theoretically would account for approximately 14 gm of calcium. Serum levels for calcium, magnesium, sodium, potassium, and phosphorus showed no marked changes throughout the study.

The patient's course in the hospital was one of gradual symptomatic improvement. He entered with considerable decompensation which subsided as the therapy progressed. The cardiac apical thrust was diminished greatly after two weeks and the patient remarked repeatedly about the joy of being rid of this annoyance. Cooperation for cardiac catheterization could not be obtained and the patient was discharged in October feeling better than he had for a long time. He was seen in the O.P.D. periodically and remained in fair condition still using digitalis and diuretics. Several months after his discharge from the hospital he met his demise. An autopsy could not be obtained.

B. Coronary Artery Disease

The first paper on the use of EDTA in instances of angina pectoris was published from Detroit in 1956. Drs. Clarke and Mosher (3) of Providence Hospital in Detroit submitted findings on a series of 20 patients who received a total of between 75 and 200 gm of Na_2EDTA. Nineteen survivors of this group experienced unusual symptomatic relief. Six patients with abnormal electrocardiograms showed changes to normal patterns during therapy.

No further report of this nature appeared in the literature until 1960. In August, 1959, at a symposium sponsored by the Hahnemann Medical College and Hospital, Drs. Meltzer, Ural and Kitchell, of Philadelphia, reported a series of 10 cases with known coronary disease characterized by intractable anginal syndrome (10). A tabulation of the findings in this experimental series is given in Table 1.

It was pointed out by the Philadelphia group that T wave changes for the better often occur without benefit of therapy and one may view this finding with skepticism. They add, however, that the reduction in the heart size of three of the patients with cardiomegaly scarcely could be due to causes other than Na_2EDTA. A single intravenous infusion in this study contained 3.5 gm of Na_2EDTA in 500 cc of 5 per cent glucose as compared to the 5 gm dose used in the series reported by Clarke *et al.* (3). It also was pointed out that the time required for symptomatic relief began 2 to 3 months following cessation of therapy. An interesting observation cited by these authors was the apparent improvement in the arthritic condition of two of the patients, one of whom had osteoarthritis and the other rheumatoid spondylitis of the cervical spines.

C. Cerebrovascular and Peripheral Vascular Disease

Cerebrovascular and peripheral vascular disease resulting from arteriosclerosis are two important areas of pathology which warrant investigation. There is conflicting evidence concerning the use of Na_2EDTA in the latter condition. In a series of more than a dozen cases of far advanced obliterative arteriosclerosis of the legs followed over a considerable period of time, it was reported that no benefit accrued to the patient. On the other hand, it was reported that in an even larger number of patients carried over a considerably longer period of time and to whom much more Na_2EDTA was given definite benefit resulted (4). It appears that response to

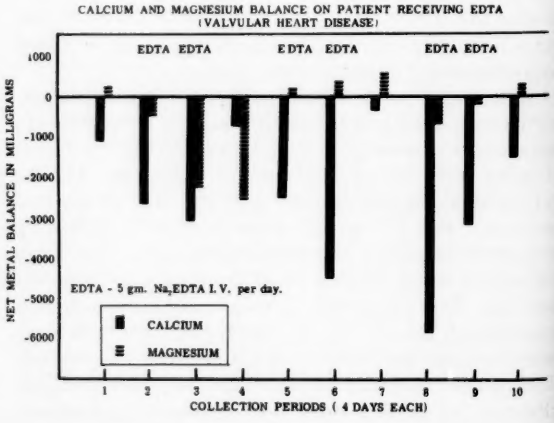


FIG. 2. Calcium and magnesium balance studies on patient W. O.

TABLE 1. *Tabulation of Response of Patients With Angina to Therapy With Na_2EDTA (10)*

Patient	Age	No. of EDTA Treatments	Total Amt., gm	History of Infarct	History of Angina	Abnormal EKG Pattern	Improved EKG Pattern	Decrease in Heart Size by X-ray	Relief of Angina
A. C.	52	49	163.5	Yes	Yes	Yes	No	—	No
L. H.	69	31	125	Yes	Yes	Yes	Yes	Yes	Yes
C. D.	63	21	57	No	Yes	No	—	—	Yes
L. W. H.	73	56	162	Yes	Yes	Yes	Yes	—	Yes
A. T.	54	21	58.5	Yes	Yes	Yes	No	—	Yes
M. A.	51	42	155	Yes	Yes	Yes	Yes	Yes	Yes
R. B.	56	20	78	No	Yes	Yes	Yes	Yes	Yes
S. G.	57	38	128	Yes	Yes	Yes	No	—	Yes
J. R.	61	22	90	No	Yes	Yes	No	—	Yes
W. H.	64	29	113	No	Yes	Yes	Yes	—	Yes

therapy is inversely proportional to involvement. The same appears to be true of cerebrovascular disease on an arteriosclerotic basis. These are areas deserving further investigation.

D. Cardiac Arrhythmias

Data are available on the treatment of cardiac arrhythmias with potassium and sodium salts of EDTA (1, 5, 7, 16, 18). These compounds appear to be of little value in supraventricular disturbances, but have a rather marked effect on ventricular arrhythmias. The therapeutic principle involves the effective binding of calcium ion in serum which is known to influence cell membrane permeability. Particularly in the presence of excess digitalis, is calcium a dangerous ion. Ventricular arrhythmias so induced may be brought under control in many instances by the use of EDTA. Some feel this to be more effective in the presence of normal serum potassium values and give K_2EDTA to make more potassium ion available to the myocardial cells.

II. CONNECTIVE TISSUE DISEASES

A. Scleroderma

Klein and Harris (8) were the first to publish on the Na_2EDTA treatment of scleroderma in November, 1955. The subject was a 45 year old female with scleroderma, sclerodactylia and calcinosis. The patient was given a total of 85 gm of Na_2EDTA over a period of approximately 55 days. Courses consisted of 7 consecutive intravenous infusions with days of rest following. Four such courses were administered. A great deal of laboratory and control work was done on this patient and the investigators are to be commended for this excellent study. Improvement was demonstrated by x-ray evidence of a marked decrease in calcific deposits and histological demonstration of regression of sclerodermatous changes in the skin. Subjectively the patient was much improved. Frequent testing of performance leaves little doubt that the physical status of this woman showed remarkable changes. It is of interest that eleven years before she had developed a progressive form of arthritis and that 5 years after this she had shown the first evidence of scleroderma, sclerodactylia and calcinosis.

Perhaps the earliest record of the treatment of scleroderma with Na_2EDTA was that of patient P. C., male, 42 years old, who entered Detroit Receiving Hospital on October 20, 1953 (Myers, G. B., Y. Morita, and A. J. Boyle, unpublished work). He was diagnosed as a case of scleroderma of several years duration. A skin biopsy of the forearm confirming the diagnosis was done at this time. On November 9, 1953, a course of EDTA was begun and continued intermittently through December 19. A total of 26 intravenous injections were administered, amounting to 130 gm of EDTA. Throughout the course of therapy, the patient was extremely loquacious and at times very difficult to manage. He complained bitterly and repeatedly of severe burning of the skin over the face and chest during treatment, and at times refused the intravenous offering. Despite patient management complications, he showed definite symptomatic relief. Movements of the hands and feet were improved noticeably, although this was felt by some of the investigators to be due to self imposed exercise. A second skin biopsy, January, 1954, revealed a calcium loss of $\frac{1}{3}$ over the initial biopsy.

Three cases of scleroderma in females were studied at the University of Wisconsin by Rukavina *et al.* (15) and by Price and coworkers (14). Both studies were reported in October, 1957. A most interesting finding in these cases pertained to tryptophan metabolism. On loading doses (2 gm orally) of L-tryptophan, the patients showed abnormally large urinary excretion of kynurenine, 3-hydroxykynurenine, kynurenic acid and N-α-acetylkynurenine. It was found that intravenous Na_2EDTA alone restored tryptophan metabolism to normal in one patient and to nearly normal in a second. The third patient was found to have normal tryptophan metabolism when given Na_2EDTA or pyridoxine.

By far the longest series of scleroderma cases was reported August, 1959, by Muller, Brunsting and Winkelmann of the Mayo Clinic (11). Their findings are summarized in Table 2. Only 9 of the 23 patients studied improved. Generally, the total dosage was conservative. The authors felt the compound to be most effective in acrosclerosis of severe degree. Like a good many other authors, this group advises the use of supplemental vitamins during treatment though they "could detect no potentiating therapeutic effect from the administration of pyridoxine and nicotinamide."

TABLE 2. *Results of Treatment With Edathamil* in Twenty-three Cases of Scleroderma (11)*

No.	Age	Sex	Diagnosis	Duration	Sever y (0–4)	Total Edathamil, gm	% Improve-ment	Complications
1	18	F	Acrosclerosis	5 yr	++++	112	50	Burning in arm; transient diplopia and dyspnea
2	59	M	Diffuse scleroderma	1 yr	++++	111	0	Slight renal irritation
3	67	F	Widespread morphea	7 mo	++	38	0	—
4	43	M	Acrosclerosis	8 yr	++++	42	0	Burning in arm
5	58	F	Acrosclerosis	5 yr	++	49	0	—
6	52	F	Acrosclerosis	1 yr	++	49	0	Burning in arm
7	31	F	Acrosclerosis	10 yr	++++	54	75	Burning in arm; slight renal irritation
8	43	F	Acrosclerosis	9 mo	++++	107	50	Burning in arm; anorexia
9	30	M	Acrosclerosis	2 yr	++++	50	75	Mild chemical phlebitis
10	57	F	Acrosclerosis	2 yr	++++	48	50	Slight renal irritation; mild chemical phlebitis; anorexia
11	47	M	Diffuse scleroderma	16 mo	++++	50	25	—
12	43	F	Acrosclerosis	1 yr	++	50	0	Slight renal irritation
13	41	M	Acrosclerosis	3 yr	++++	49	0	—
14	49	F	Acrosclerosis	1 yr	++	50	25	Burning in arm
15	26	F	Acrosclerosis	2 yr	++++	45	0	Slight renal irritation
16	34	F	Acrosclerosis	1½ yr	++++	50	0	—
17	53	F	Acrosclerosis	8 yr	++++	47	25	—
18	54	F	Acrosclerosis	7 yr	++++	49	0	—
19	27	F	Acrosclerosis	6 mo	++	39	0	Mild renal irritation
20	12	F	Acrosclerosis	2 yr	++++	40	50	—
21	48	M	Acrosclerosis	2 yr	++++	50	0	—
22	36	F	Acrosclerosis	2 yr	++++	37	0	Marked renal irritation and pustular dermatitis of mouth
23	40	F	Acrosclerosis	18 yr	++++	50	0	—

* Na_2EDTA.

B. Calcinosis Universalis

A most extensive case of calcinosis universalis in a 12 year old girl was reported in November, 1959, by Davis and Moe (6). The weight of the child when admitted to the University of Texas Hospital on April 2, 1958, was 15.4 kg and her height 133.5 cm. X-ray revealed extensive calcification in both muscles and subcutaneous tissue. She was fed through a nasogastric tube since dysphagia was so marked that she made no attempt to swallow even her own saliva. Among the various medications given was 10 mg of prednisone every 8 hours. No improvement on this therapy was evident after one month. On June 2, 1958, the intravenous administration of 0.85 gm of Na_2EDTA was started and given 5 days a week for 3 weeks. Ten days after commencement of this therapy, the nasogastric tube was removed and the child ate fairly well. From here on the hospital course was one of improvement. On August 29, 1958, another 3 week course of Na_2EDTA was begun. The patient was discharged from the hospital on October 6, 1958. On December 10, 1958, she was seen as an outpatient. She was attending school. Her weight had increased to 23 kg and height to 135.8 cm. X-ray examination showed a marked decrease in soft tissue calcification.

C. Dermatomyositis

There are two cases of dermatomyositis known to us which have been treated with Na_2EDTA. One has been reported by Pollock (13), but follows a pattern of mixed therapy and very low dosages of Na_2EDTA. The other, a case of an 8 year old girl, was treated by our group in Detroit for approximately one month during which time it became possible for her to feed herself, to raise her arms above her head, as well as to walk about the room. We lost sight of her for almost a year. Our attention was recalled to this case by an article in the daily newspaper which described her cure as nothing short of a miracle and associated it with the composition of a religious hymn.

D. Rheumatoid Arthritis and Spondylitis

Because most of the situations which have been cited are in the literature, no attempt was made to discuss more than generalities concerning the points of interest as the result of Na_2EDTA therapy. The matter of rheumatoid arthritis, however, is something new. Three out of some ten patients studied will be presented in some detail as well as one case of early rheumatoid spondylitis. No medication other than Na_2EDTA was given in this series once therapy began.

Case I. S. B., a 41 year old male, was admitted to the hospital on 8/27/56 with painful swelling of the hands, feet, knees and neck and with a temperature of 101 °F. The patient complained of severe pain in the left hip and back. One week earlier, following an upper respiratory infection, he was seized suddenly with an excruciating

back pain radiating down both legs posteriorly. He was given a dose of penicillin by his physician. Two days later, the pain became so accentuated that he no longer could walk and was forced to go to bed. The following day, there was generalized swelling and stiffness of hands, feet, knees and neck. A history of frequent lumbosacral pain and a long standing prostatitis were the patient's only significant illnesses. Psychologically, he was depressed over the death of his wife which had occurred about three months before. His craft was that of a special metal alloys maker which subjected him to extremes of temperature and much heavy lifting.

Physical examination revealed a well developed, well nourished male in acute distress. There was generalized joint swelling and tenderness with severe limitation of motion. Passive manipulation of the joints elicited pain. The neck was stiff and painful. The pharynx, chest, heart and abdomen were normal. Rectal examination revealed a grade 2 prostatic hypertrophy.

Laboratory. Red blood cell count (RBC), 4.3 million per cu mm; white blood cell count (WBC), 9,000 per cu mm; 72 per cent neutrophiles; 27 per cent lymphocytes; 1 per cent monocytes. Sedimentation rate: 61 mm hr; EKG normal; X-ray: chest normal; spine, minimal narrowing of second lumbar interspace with localized hypertrophic arthritic changes; hands, no evidence of arthritic changes; Urinalysis: normal.

Hospital course. The patient was given sulfadiazine and a five day test of salicylates, 30 gr three times a day. There was no subjective or objective improvement in the patient's condition. A diagnosis of acute rheumatoid arthritis was made at this time and the patient was given prednisone in 5 to 15 mg doses three times a day following which there was relief of pain. His temperature continued to spike daily, however, and there was little if any subsidence of the joint swelling and stiffness. On 9/27/56, or one month after hospital admission, the patient was referred to the research department. Daily intravenous administration of 2.5 gm of Na_2EDTA in 500 ml of distilled water given over a period of one hour was begun. All other medication was discontinued at this time. For the first few days, the patient became noticeably worse. Joints became stiffer and more painful. The patient complained bitterly of his worsened condition. His sedimentation rate at this time was 104 mm/hr. From 9/27/56 to 10/15/56 he received 15 intravenous infusions. At the end of this period, the joint pain and swelling had subsided remarkably and the patient was ambulatory. Limitation of joint motion was still marked and pain could be elicited on passive manipulation of the extremities. Back pain had subsided almost completely. The patient's temperature had returned to normal and he was discharged from the hospital on 10/15/56. A second intravenous course of Na_2EDTA was begun 10/29/56 on an outpatient basis and carried through 11/16/56, during which time he received 15 more intravenous infusions. The sedimentation rate on 11/15/56 was 34 mm/hr. The residue at this time was some stiffness of the fingers. He returned to work early in December, 1956. On 12/14, 12/21 and 12/28/56, respectively, he received 2.5 grams of Na_2EDTA. The sedimentation rate on the last date was 25 mm/hr. No further treatment was given. On 2/6/57 the sedimentation rate was 8 mm/hr. To date the patient has been free of his former difficulty. He has been seen periodically by a member of this department. No medication of any sort has been prescribed since the last intravenous infusion of Na_2EDTA on 12/28/56. The patient response in this instance was sufficiently impressive to warrant a more complete study before and during the course of therapy in future cases.

Case II. The following deals with the study and treatment of an early case of rheumatoid arthritis in a female 31 years of age. In addition to conventional diagnostic laboratory procedures a 24 hour tryptophan loading test was performed.

The patient was first seen on 11/7/58. Her chief complaint was swelling of the ankles associated with pain and stiffness on motion. She stated that earlier in the week there had been soreness in both knees, the left being slightly swollen. At this time, the knees were somewhat improved while the ankles became progressively worse. She also complained of discomfort related to the left eye. Past history revealed that four years before she had been forced to quit work for a month because of fatigue. At the end of this period, she developed an acute iritis which resulted in hospitalization. She remained in the hospital for one week during which time she was treated with ACTH. She was then permitted to go home but continued on ACTH for approximately three weeks more. Undiagnosed left eye discomfort recurred during the summer of 1957. She visited an ophthalmologist and was given hydrocortisone ophthalmic ointment for local application. There was an uneventful recovery. During these two episodes, there were no manifestations of joint involvement. Further questioning revealed that the first symptoms of the present illness became noticeable with the onset of her menstrual period. She could not relate the earlier episodes with this time of the month. Two weeks prior to the present illness she was aware of a sore throat which subsequently developed into a slight cold. She complained of not feeling rested despite more than the usual amount of sleep.

Physical examination revealed a well nourished subject of slender build. Except for the following observations the examination was essentially negative. The left conjunctiva was injected. No other abnormalities were found on ophthalmoscopic examination. The hands of the patient were moist and cold. No signs of joint involvement were noted. There was copious axillary perspiration. Subjective complaint was elicited on manipulation of the knees, though swelling was not present. Both ankles were grossly swollen and noticeably warm to the touch. There was definite limitation of motion. Passive manipulation and pressure appeared to be painful. Palpation revealed an accumulation of fluid bilaterally. The oral temperature was 98.8°F.

Laboratory findings. Hemogram: Hemoglobin, 13.6

gm/100 ml; RBC, 4.28 million/cu mm; hematocrit, 40 ml/100 ml; WBC, 9400/cu mm; Differential: neutrophils 77 per cent filamented; 4 per cent nonfilamented; lymphocytes, 16 per cent; monocytes, 2 per cent; basophils, 1 per cent. An antistreptolysin titer showed 12 Todd units. Sedimentation rate, 35 mm/hr; C-reactive protein, 3+. R.A. (latex) test was positive. Urinalysis was negative. An electrocardiogram showed a heart rate of 90 with a PR interval of 0.17 second. The T wave was inverted in Lead III.

Contemplating treatment of this patient with disodium EDTA, it was decided to do a test of salicylate therapy to assist in ruling out possible rheumatic fever. Accordingly, she was confined to bed for one week and given 60 grains of sodium salicylate daily. At the end of this period there was no joint improvement and the sedimentation rate on 11/14/58 was 36 mm/hr. All medication was discontinued. From 11/16/58 to 11/17/58 a 24 hour specimen of urine was collected. On 11/17/58 the patient was given a 2 gm dose of L-tryptophan. Urine was then collected for the succeeding 24 hours. Both these specimens then were analyzed chromatographically for tryptophan metabolites. On the loading test both kynurenine and 3-hydroxykynurenine were found to be elevated over normal values. On completion of therapy these values returned to normal as shown in Table 3.

TABLE 3. *Tryptophan Loading Test**
Micromoles of Metabolite Excreted per 24 Hours

Metabolite	For 24 Hour Urine Ending				Normal Response, Range and (Average)	
	Basal day 11/17/58	After tryptophan 11/18/58	Basal day 1/15/59	After tryptophan 1/16/59	Basal day	After tryptophan
Pyridone	91	133	67	69	34–149 (95)	74–213 (135)
Kynurenic acid	11	69	10	35	2–19 (11)	30–99 (60)
Xanthurenic acid	5	24	4	16	4–15 (9)	18–71 (39)
Indican	339	257	126	136	175–669 (387)	167–760 (412)
Anthranilic glucuronide	7	6	2	4	2–9 (5)	2–19 (8)
o-Aminohippuric acid	27	54	14	29	15–36 (25)	23–94 (47)
Acetylkynurenine	10	27	4	8	6–22 (12)	8–32 (18)
Kynurenine	17	120	11	52	6–21 (13)	9–83 (28)
3-Hydroxykynurenine	38	197	9	46	11–61 (28)	7–76 (52)
4-Pyridoxic acid	2.40	3.24	2.40	2.40		

* The analyses for tryptophan metabolites were done by Drs. J. M. Price and R. R. Brown of the Cancer Research Hospital, University of Wisconsin.

On 11/18/58 the first intravenous infusion of 2.5 gm of Na_2EDTA (pH 7.4) in 500 cc of 5 per cent glucose was administered. The dropping rate was fixed to accomplish the total infusion in 75 minutes. The patient was given Na_2EDTA five times per week for three weeks and then permitted one week of rest. This first course constituted 15 infusions or a total of 37.5 gm. From 12/15/58 to 1/14/59 another 15 intravenous infusions were given to make a total of 30. During this latter period the patient became symptom free. No other medication or physical therapy was resorted to at any time. The sedimentation rate on 1/23/59 was 9 mm/hr. The C-reactive protein was found to be negative on 12/17/58 and also on 1/14/59. Except for an occasional trace of albumin, a total of 14 urinalyses were essentially negative. Serial starch gel electrophoresis patterns of serum showed a progressive decrease in the prealbumin fraction during the course of therapy.

After 21 infusions the patient's white count dropped suddenly to 3600/cu mm with 10 per cent monocytes. One week later it had returned to normal. A similar picture was seen on 1/12/59. By 1/19/59 the total white count approached normal though the differential remained abnormal. Microscopic examination of the cells showed a borderline abnormal morphology for the lymphocytes. No definitive conclusions could be reached. On 1/23/59 the total white count was 5000/cu mm with the differential improving. There was slight increase in granulation of granulocytic cytoplasm. The lymphocytes appeared normal. In our rather wide experience with Na_2EDTA administered in both animals and humans, this blood picture is unique.

Throughout the period of treatment the patient had two menstrual periods. With the period occurring on or about 12/2/58, there seemed to be a slight exacerbation of ankle stiffness which was noted most on dorsiflexion. All swelling had not yet disappeared. By the next period on 12/29/58 all swelling had disappeared and extreme dorsiflexion of the ankles was necessary to produce discomfort.

By 3/5/59 no abnormalities were detectable. A slight exacerbation of ankle swelling was noted on 11/24/59. The sedimentation rate was 35 mm/hr. From 11/24/59 to 12/24/59, a series of 20 intravenous infusions were administered. The sedimentation rate on 12/21/59 was 11 mm/hr.

Throughout the first 5 months of this year, the patient was in excellent health and free of all symptoms. Early in June she suffered a severe bout of diarrhea, after a week of which an intense exacerbation of her arthritis symptoms recurred. She was seen on 6/13/60 by which time both ankles, knees, as well as the left shoulder, elbow and hand were involved. The sedimentation rate was 67 mm/hr, temperature 102°F. Na_2EDTA infusions were started using a 2 gm per day dosage. During the first week of therapy the symptoms became worse and with this the sedimentation rate climbed to 107 mm/hr by June 16. From there on improvement occurred gradually. By 8/1/60 the patient

had resumed a full schedule of work. For the ensuing 30 days improvement in physical appearance and activity returned to normal rapidly. By 8/22/60 the patient had received 38 infusions of Na_2EDTA and therapy was discontinued.

Case III. D. K., a boy 9 years of age, was first seen in our department in December 1959. A summary of his case history obtained from another hospital was as follows.

He was admitted to the hospital July 13, 1959, because of migratory arthralgia. On July 31, 1959, he was discharged with a tentative diagnosis of Henoch-Schönlein's syndrome. He had done fairly well but continued to have an intermittent fever. A week prior to a second admission on 9/30/59 he developed a rhinorrhea and his temperature rose to 104°F. Sedimentation rate at this time was 91 mm/hr. He was given aspirin during the few days before hospital admission.

Physical examination revealed a 9 year old, well nourished, well developed male. Throat clear, no stiffness of the neck, and lungs normal to auscultation and percussion. The heart rate was 140, sounds were muffled, a grade 2 systolic murmur was heard best between the apex and sternum. The murmur was not transmitted to axilla. The abdomen was soft. No masses were palpable. There was slight swelling of both wrists and pain on extension of the right wrist. EKG and chest x-ray on October 1 were normal. The antistreptolysin titer was 625 Todd units; C-reactive protein, 4 units; WBC, 13,650/cu mm; Hb, 8.6 gm/100 ml. X-ray of the right wrist showed no arthritic changes. After consultation it was decided that the spiking temperature curve, despite aspirin in adequate quantities, strongly suggested rheumatoid arthritis. The murmur was considered functional.

Hospital course. The patient was given 30 mg of Aristocort daily. This dosage was reduced one week later. Subsequently, the temperature rose and the right wrist became more painful. The Aristocort was increased and 200 mg of Plaquenil sulfate twice daily was added to the regimen. The sedimentation rates on 10/6, 10/12 and 10/20 were 46, 21 and 41 mm/hr, respectively. There was a gradual decrease in temperature and sedimentation rate from this point on. During the next two weeks, the steroid was reduced slowly to 10 mg per day and the Plaquenil continued. The patient was discharged on 11/6/59 to his private physician.

Figure 3*A* is a photograph of the patient taken after his release from the hospital as he appeared when first seen by one of us. His physician at this time had decided to reduce the Aristocort still further and finally discontinue it altogether. This was done over a period of weeks. By 1/25/60 symptoms had recurred. The sedimentation rate was 61 mm/hr. On 1/26/60 intravenous Na_2EDTA was administered in a dose of 1 gm in 250 ml of 5 per cent glucose. From 1/26/60 to 2/4/60 the patient was given 9 intravenous infusions and treated as an outpatient. He then was given a ten day rest period. Between 2/15/60 and 2/24/60 he was given 8 more infusions. By 3/11/60 he had received a total of 26 and for a time was given one intravenous infusion weekly. The patient had been rated in school as a rather dull subject. It is interesting that this picture has changed. He is in the 5th grade, presently, and is considered about average. Despite the fact that his course has been one of slow but steady improvement, both subjectively and objectively, he still has occasions when his temperature rises and there is sporadic joint swelling with mild discomfort. On March 1, 8, 14, 27, 31 and April 3, 7, 11, 12, 13 his temperature was just above 100°. On the other days in March and April it was normal (taken AM, M, PM). The sedimentation rate reached a high of 86 mm/hr on 2/1/60. On 4/19/60 x-ray of wrists and hands showed no arthritic changes. The WBC was 10,700/cu mm with 76 per cent neutrophils, 23 per cent lymphocytes, 1 per cent eosinophils. Hemoglobin was 12.0 gm/100 ml, C-reactive protein 4 units, R. A. (latex) negative. The sedimentation rate was 53 mm/hr and the antistreptolysin titer 160 Todd units. The complete blood count was normal. On 5/3/60 the boy was given an injection of Bicillin as well as his weekly one gm infusion of Na_2EDTA. By 5/10/60 the sedimentation rate had dropped to 8 mm/hr, the temperature to normal and there have been no indications of swelling or discomfort. Throughout this summer the sedimentation rate has fluctuated, the C-reactive protein has remained 4+ and the antistreptolysin titer 160 Todd units. The parents report the patient is extremely energetic and great difficulty is encountered in restricting activities.

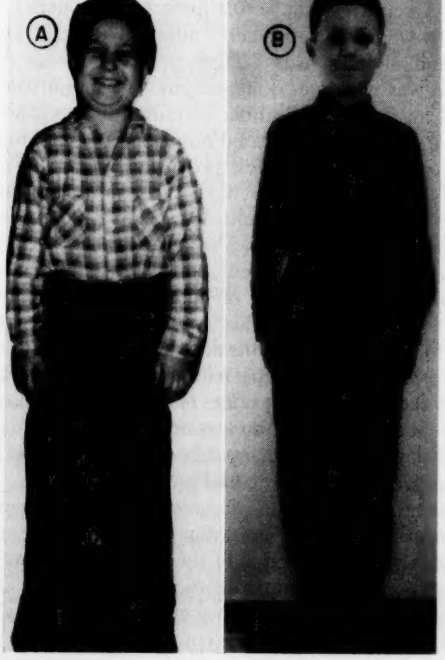


FIG. 3. *A*: Patient D. K. on corticoids; *B*: Patient D. K. after Na_2EDTA therapy.

He has had no additional Na_2EDTA and is being seen about twice a month. Figure 3*B* is a photograph of the child after he received his last Na_2EDTA infusion.

Case IV. H. B. a male, 46 years of age, was first seen in our department in May, 1957. The preceding January he had been admitted to another hospital for diagnosis. The summary of the findings is as follows.

This 46 year old white male was seen originally for complete examination approximately 3 weeks prior to his admission to the hospital. At that time he had complaints of pain in the back, not localized, and some associated pains in the chest; a loss of weight of 15 lbs in the past few months; a low grade temperature elevation; and a generalized feeling of being run down. The patient was seemingly in no acute distress, he was a thin white male without remarkable physical findings, except those pointing to the spine, of which function of the spine was mildly limited. The patient had x-rays as an outpatient and the spine did show some osteoporosis. The patient was admitted to the hospital on 1/30/57 for further work-up.

The white blood count was 14,750/cu mm, hemoglobin 13.4 gm/100 ml. The hematocrit was 43 per cent, the differential showed 2 band cells, 78 polymorphocytes, 19 lymphocytes and 1 monocyte. The urinalysis was not remarkable. The serum proteins on admission showed a total of 8.9 gm per cent, with a serum albumin of 5.0 gm per cent, and serum globulin of 3.9 gm per cent. The alkaline phosphatase was .9 units. Inorganic phosphorus was 3.9 mg per cent, serum calcium was 11 mg per cent. The sedimentation rate was 110 mm per hour. A repeat of the serum proteins was done. Total proteins were 6 gm per cent, albumin was 4.8 gm per cent and globulin was 1.2 gm per cent. Bence Jones' protein was negative. Bone marrow aspiration study revealed an increased number of immature myeloid cells and the presence of increased numbers of esoinophils and eosinophilic promyelocytes, probably indicating a subsiding infection. However, there was no diagnostic pattern of especially mild multiple myeloma. X-rays of the thoracic and lumbar spines were compatible with rheumatoid type of involvement, particularly suggested by the marked straightening of both thoracic and lumbar curves, though no evidence of bone involvement was thought to be present at this time. Skeletal survey showed slight osteoporosis and coarsening of the trabecular pattern in the spine and flat bones of the pelvis, but no additional specific abnormality was noted.

The patient was discharged home with the final diagnosis on 2/6/57 of rheumatoid spondylitis (early).

At the time he came to us, there had been an exacerbation of back pain and limitation of movement. X-ray of the spine revealed essentially the same findings as those obtained on his hospital admission a few months before. After some deliberation, we began a course of intravenous Na_2EDTA on 5/21/57 which continued through 5/31/57 during which time he received 9 infusions of 3 gm each. At this time there was subjective relief from back pain. A second course of the same dosage was given between 6/10/57 and 6/28/57, totaling 15 intravenous infusions. There was definite objective improvement at the end of this series and a third course followed consisting of 10 intravenous infusions between 7/8/57 and 7/19/57. Seven sedimentation rates covering the period of therapy were all around 20 mm/hr.

At this time, 8/21/60, the patient's weight has increased 13 pounds and he is able to touch the floor with his finger tips without bending his knees. Our x-ray on 5/20/57 revealed generalized osteoporosis of the entire bony structure of the lumbar spine and pelvis, particularly in the area of the sacroiliac joints. There was also marked straightening of the lumbar spine due perhaps to underlying muscle spasm. On 4/21/60 another x-ray of the spine showed minimal straightening of the lumbar spine with no extension of the osteoporosis. The sedimentation rate was 9 mm/hr and the blood titers were normal.

DISCUSSION

It is hardly necessary to point out that Na_2EDTA is not a curative drug. It is, however, rather unique in that it attacks fairly consistently some of the most refractory diseases in man. In our opinion it deserves a place in Medicine's therapeutic armamentarium. In addition, the compound generates hope of a more complete understanding of the disease process. At this writing no definitive action of EDTA in circulatory and sclerosing diseases has been established. In the obvious metal poisoning, it is apparent that removal of the metal results in improvement. The use of calcium EDTA is well known to all of us as the treatment of choice in lead poisoning and serves as a most excellent and specific example. The role of Na_2EDTA in the degenerative diseases which have been mentioned in this discourse is not explained so simply. Let us examine some of the possibilities.

Lowering of Serum Ionic Calcium

In the early days of our experience with Na_2EDTA, we felt that an important part of the effect of the compound might be attributed to the transient state of serum calcium ion deficiency which permitted physical solution of metastatic calcium deposits from various areas in the body. Despite the large supply of labile calcium in bone, all blood does not go directly to bone where it immediately would become saturated fully with this ion. That which did not, or the major part, remained free to establish calcium equilibrium with tissue. The net result of this solution action would be increased calcium turnover and loss with particular emphasis on the turnover.

Increased Parathyroid Function

It is conceivable that the lowering of ionic calcium in the blood, brought about by Na_2EDTA, could produce

a parathyroid response within physiological limits which would result in a more dynamic exchange of calcium throughout the organism. Such exchange would eventually lead to increased calcium loss and a reduction of net metastatic calcium.

Trace Metal Balance

The Na_2EDTA effect on trace metals would be one of redistribution or elimination or both. This was demonstrated in rats with manganism some years ago using $CaNa_2EDTA$ (9). The enhancement or inhibition of enzyme systems then might be responsible in a rather direct way for the observed changes in a given pathological state.

Direct Action of EDTA Molecule

From what is known of this compound, its action, once satisfied with a metal, would appear to be rather minimal. Schroeder and Perry (17) have demonstrated in rats the effect that EDTA metal complexes have on hypertension but consider these results directly related to the binding capacity of EDTA. In other words, the more stable the metal chelate the less the response in the hypertensive animal. These two workers and others have demonstrated a lowering effect by EDTA on serum cholesterol when given intravenously or orally (12). These effects are usually transient and former levels promptly obtain after discontinuing its use. It would seem more reasonable that whatever EDTA does is indirect rather than due to the molecule *per se*.

If the answer to the effect of EDTA is to be found in its relation to trace metals in the wrong areas or in quantitative amounts which render them ineffectual, the causative relations may be, indeed, difficult of solution. It does not seem likely that, through the long experience of evolution, nature would leave so many of us easy prey to trace metal imbalance and, in addition, discriminate between victims on the basis of sex. To be sure, many instances of metal poisoning are known and their characteristics charted. It is true that toxicity and distortion of normal metabolic function are brought about by those metals which we designate as trace under ordinary circumstances. When poisoning from such metals occurs, they are to be found in quantities many fold over that normally present in the body.

It seems reasonable that by using the calcium chelate of EDTA as therapy for the diseases we have discussed instead of Na_2EDTA the real importance of trace metals in these conditions might become apparent. But let us examine another moiety which would appear to have a far greater influence on metabolism than the ubiquitous trace metals. Consider for a moment the prodigious effect of adrenalin. If anyone doubts the power of this hormone let him submit to an injection of 1 milligram. Metabolic rates are elevated and, in most instances, nervousness and apprehension will be apparent. There has been in all probability no change in trace metal balance during this experience. If one administers enough thyroid hormone to raise the protein bound iodine of the myxedematous subject from two to six microgram per cent, the enlarged heart will become normal in size and the myxedematous skin disappear. The otherwise premature atherosclerosis will be halted and the mental activity restored. The slow moving, rather dull individual will again become a member of society. This change has not been brought about by trace metals except as they function in the wake of a more potent entity. And what of insulin, the lack of which speeds deterioration in every conceivable direction? The potency of the corticoids is well known, the absence of which grossly produces shock and death, and the abundance of which results in such rapid gluconeogenesis that bone structure may be destroyed.

One might go on at considerable length in this vein with many more examples, none of which would require, at least within our knowledge, more than the average quantity of trace metals. But metals known to us, which one could scarcely distinguish as present in trace amounts, do evoke, in ionic form, dramatic metabolic and physiological action. The concentration of sodium ion is highly provocative in its influence on the antidiuretic hormone. Ionic calcium (and there is some evidence that phosphate ion) controls parathyroid activity. Indeed, it may be that the chief difference in time manifestation of atherosclerosis in the sexes is due to the more frequent stimulation of parathyroid activity in the female. Estrogen enhances the storage of nitrogen and calcium and is conducive to osteoblastic activity in bone. Parathormone effects the mobilization rather than storage of calcium and is known to attack bone matrix. The years between menarche and menopause are characterized by monthly fluctuations in estrogen elaboration. What synergism or antagonism exists between estrogen changes and parathormone which would keep calcium metabolism dynamic? During pregnancy histological evidence of parathyroid hyperplasia has been demonstrated. In the pregnant rheumatoid it is possible that this gland has a role to play in the remission of symptoms as well as the corticoids.

It would be ludicrous to believe that the diseases we have discussed are solely metal disturbances. One does not deposit nor get rid of structural metals without gain or loss of proteins and mucopolysaccharides. Mesenchyme tissue is everywhere and if EDTA in one form or another is efficacious in the metabolism of this most important fraction of the organism, it is no little wonder that therapeutic manifestations seem at times unrelated. It would seem reasonable to suggest that EDTA function has been demonstrated to have its greatest effect on "calcific" or sclerosing diseases and this effect is mediated, at least partly, through parathyroid activity brought about by intermittently lowering serum calcium ion concentration.

REFERENCES

1. Bechtel, J. T., J. E. White and E. H. Estes. *Circulation* 13: 837, 1956.
2. Boyle, A. J., H. S. M. Uhl and H. H. Brown. *Am. J. Clin. Path.* 23: 801, 1953.
3. Clarke, N. E., C. N. Clarke and R. E. Mosher. *Am. J. M. Sc.* 232: 654, 1956.
4. Clarke, N. E., N. E. Clarke, Jr. and R. E. Mosher. *Am. J. M. Sc.* 239: 732, 1960.
5. Cohen B. D., N. Spritz, G. D. Lubash and A. L. Rubin. *Circulation* 19: 918, 1959.
6. Davis, H. and J. P. Moe. *Pediatrics* 24: 780, 1959.
7. Kabakow, B. and M. J. Brothers. *A.M.A. Arch. Int. Med.* 101: 1029, 1958.
8. Klein, R. and S. B. Harris. *Am. J. Med.* 19: 798, 1955.
9. Kosai, M. F. and A. J. Boyle. *Indust. Med.* 25: 1, 1956.
10. Meltzer, L. E., E. Ural and J. R. Kitchell. In: *Metal-binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 132.
11. Muller, S. A., L. A. Brunsting and R. K. Winkelmann. *A.M.A. Arch. Dermat.* 80: 187, 1959.
12. Perry, H. M. and H. A. Schroeder. *J. Chron. Dis.* 2: 520, 1955.
13. Pollock, R. C. *California Med.* 90: 284, 1959.
14. Price, J. M., R. R. Brown, J. G. Rukavina, C. Mendelson and S. A. M. Johnson. *J. Invest. Dermat.* 29: 289, 1957.
15. Rukavina, J. G., C. Mendelson, J. M. Price, R. R. Brown and S. A. M. Johnson. *J. Invest. Dermat.* 29: 273, 1957.
16. Sapeika, N. *Arch. internat. pharmacodyn.* 97: 373, 1954.
17. Schroeder, H. A. and H. M. Perry. *J. Lab. & Clin. Med.* 46: 416, 1955.
18. Surawicz, B., M. G. MacDonald, V. Kaljot and J. C. Bettinger. *Am. Heart J.* 58: 493, 1959.
19. Uhl, H. S. M., H. H. Brown, A. Zlatkis, B. Zak, G. B. Myers and A. J. Boyle. *Am. J. Clin. Path.* 23: 1226, 1953.

DISCUSSION

Metals and chelating agents in relation to atherosclerosis

R. GORDON GOULD

Stanford University School of Medicine, Palo Alto, California

All of us working in the field of cholesterol metabolism have followed with great interest the work of Dr. Schroeder and Dr. Perry on the effect of EDTA in decreasing plasma cholesterol levels and the work of Dr. Curran and others on the effect of vanadium salts in decreasing the concentrations and the rates of biosynthesis of cholesterol in various tissues. Perhaps it would be appropriate to say a word about why this particular aspect of trace metal biology is of such great interest and importance. Practically everyone in this country, and in other countries with a high standard of living, gradually accumulates in his arteries lesions which contain high concentrations of cholesterol and phospholipids. These lesions, even in individuals with no history or clinical evidence of vascular disease, are frequently so widespread and advanced as to result in an extremely high concentration of cholesterol in the whole artery. For example, Dr. Wissler and Dr. Jones of the University of Chicago and I recently observed increases of up to 20 to 30 times the normal value in the cholesterol concentration in whole aorta, iliac and coronary arteries in patients who had died from such diseases as chronic glomerulonephritis and carcinoma of the breast. Calcium also accumulates but it is generally agreed that this is secondary to the atheromatous changes associated with cholesterol deposition.

The factor or factors responsible for the process of atherogenesis are still unidentified but a majority of investigators believe that decreasing the plasma cholesterol level is the best lead we have at the moment to the prevention of this extraordinarily prevalent disease. There are two types of approach to this general problem. One is to try to block the synthesis of cholesterol, either in the liver or in the body as a whole, so as to deplete the body of its stores of cholesterol and thus lower the plasma level. However, lowering the rate of synthesis will not necessarily or automatically lower the plasma level. In hypothyroidism it has been shown that there is a decreased rate of synthesis and, yet, there is an increase in the plasma level. This may be due, in part, to the fact that there is also a decrease in the rate at which cholesterol is utilized and excreted so that the total amount of cholesterol in the body does not decrease in spite of the lower rate of synthesis; but there are undoubtedly other factors operating to increase the plasma level. However, it frequently is true that decreasing the rate of biosynthesis will result in decreasing the plasma level, as Dr. Curran has shown with vanadium administration and as Blohm

et al. (1) have shown to result from the new drug MER 29 (Triparanol).

It is of interest to note that the various inhibitors of cholesterol biosynthesis do not operate on the same step. The over-all rate of the whole process from acetate to cholesterol may vary enormously in liver. It can be decreased to almost zero by feeding cholesterol or by a number of procedures which result in an increase in the concentration of free cholesterol in liver. It can be increased by whole body x-irradiation, by intravenous injection of Tween or by other procedures which decrease the liver cholesterol concentration. The locus of this type of regulation of cholesterol biosynthesis, which we may define as physiological control, is shown in Figure 1.

This figure is a condensed scheme of the more than twenty-five reactions known to be involved in cholesterol biosynthesis. These large changes in rate are not observed if mevalonic acid is used as a cholesterol precursor instead of acetate, indicating that the control lies between acetoacetyl CoA and mevalonic acid, (3). Bucher *et al.* (2) have reported evidence that the principal controlling reaction is the reduction of β-hydroxy-β-methyl-glutaryl CoA to mevalonic acid, and this conclusion has recently been confirmed by Siperstein (4). All the intermediates before mevalonic acid are metabolized by other pathways as well and would not be expected to accumulate in case of a block in cholesterol biosynthesis. Furthermore, the reactions in this part of the pathway are all reversible. The reduction to mevalonic acid is the first irreversible step. Mevalonic acid is converted into cholesterol with ten to twenty times the efficiency of acetate. Small amounts of branch chain fatty acids are apparently formed from it but in general it goes to cholesterol irreversibly and almost inevitably. It is evident that the reduction of HMG to MVA is the ideal step to control but none of the drugs available at present appears to work on precisely this step. α-Phenylbutyrate inhibits the conversion of acetate to acetyl CoA and thus interferes with all pathways of acetate metabolism. Vanadium acts between mevalonic acid and squalene, as Dr. Curran has described, quite possibly by competing with manganese or magnesium that is required for the conversion of mevalonic acid to isopentenylpyrophosphate by two successive reactions with ATP.

MER 29 blocks very specifically the conversion of desmosterol to cholesterol resulting in the accumulation of desmosterol and possibly other sterol intermediates. However, the total sterol concentration does not rise above normal and in fact is usually decreased, in some

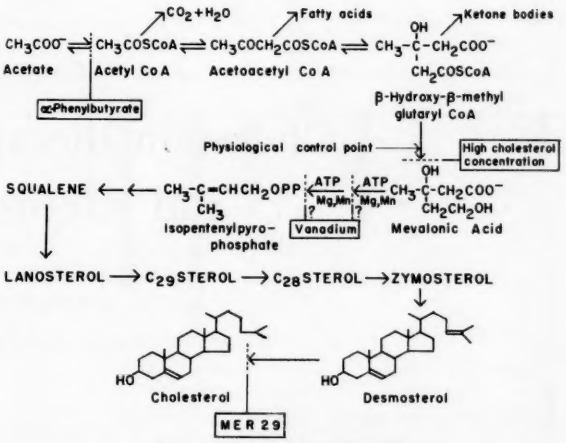


FIG. 1. Cholesterol biosynthesis.

tissues very strikingly. MER 29 is of particular interest because it lowers the concentration and apparently also the rate of biosynthesis in many extrahepatic tissues as well as in liver.

The second and more commonly used approach is to search for empirical methods of decreasing the plasma cholesterol level without considering any changes in the rate of biosynthesis or in tissue concentrations. Several methods of producing a moderate decrease in plasma level have been reported, including the use of EDTA, but little is known about the mode of action of any of them. EDTA does not appear to have an appreciable effect on biosynthesis or concentration in tissues. In fact, it is frequently added to homogenates to keep trace metals from interfering with cholesterol biosynthesis. It may act by decreasing the capacity of plasma to carry cholesterol. Since cholesterol is almost completely insoluble in water, it can only exist in plasma combined with a specific kind of protein.

The linkage between lipids and protein in lipoproteins is a fascinating riddle and is quite possibly the key to the question of how the plasma cholesterol level is regulated. It is evident from the structures of cholesterol, cholesterol esters, phospholipids and triglycerides that no covalent or chelate bonds are involved so we are left with the idea that a large amount of hydrophobic lipid must in some way be hydrogen-bonded to a small amount of protein to yield a freely water-soluble lipoprotein. Future investigation of this problem may well be as revealing and as far reaching in its implications as the studies on chelation have been in the recent past.

REFERENCES

1. BLOHM, T. R., T. KARIVA, M. E. LAUGHLIN AND F. P. PALOPOLI. *Fed. Proc.* 18: 369, 1959.
2. BUCHER, N. L. R. In: *Ciba Foundation Symposium on the Biosynthesis of Terpenes and Sterols*, edited by G. E. W. Wolstenholme and M. O'Connor. Boston: Little, 1959, p. 46.
3. GOULD, R. G. AND G. POPJAK. *Biochem. J.* 66: 51P, 1957.
4. SIPERSTEIN, M. D. AND M. J. GUEST. *J. Clin. Invest.* 39: 642, 1960.

DISCUSSION

Chelation therapy in circulatory and sclerosing diseases

H. MITCHELL PERRY, JR.[1]
Washington University School of Medicine, St. Louis, Missouri

In addition to metal poisoning, where the rationale for using metal-binding agents is obvious, a great many diverse human ills have been treated with chelating agents, particularly ethylenediaminetetraacetic acid (EDTA). For many of these conditions, it seems unlikely that EDTA eventually will prove to be the therapy of choice. At present, however, there are not enough data to warrant comment except in a few categories of disease.

Dr. Boyle has just discussed the results of administering EDTA to patients with a variety of "circulatory and connective tissue diseases." My only pertinent comments regarding the latter are to recall that Dr. Price has ably presented the available information pertaining to scleroderma elsewhere in this symposium and to remark that the literature has little to add to Dr. Boyle's discussion of chelate therapy for the other three syndromes: calcinosis universalis, dermatomyositis, and rheumatoid arthritis. I shall be almost as brief regarding two of the four "circulatory diseases" which were mentioned. Additional information about the effects of EDTA in valvular heart disease is limited to the statement by Meltzer, Ural, and Kitchell that "calcification of the aortic valve and ring [in one patient] . . . showed an apparent decrease" following the administration of EDTA (3). In contrast, there is considerable evidence that EDTA often can improve temporarily ventricular arrhythmias; moreover, it seems very likely that the effect is mediated by the binding of circulating ionic calcium.

Some further discussion of atherosclerosis seems warranted by the unexplained beneficial effects reported in this very serious and prevalent condition. Dr. Boyle and his coworkers feel that significant improvement has occurred in most of their patients who received numerous daily parenteral injections of 5.0 gm of the neutral (pH 7.5) sodium salt of EDTA for angina pectoris. When Meltzer and his coworkers attempted to confirm these observations, using somewhat smaller doses of similar EDTA, they discontinued treatment at the end of three months because of disappointing results; but, three months later, they felt that improvement had occurred in nine of their ten patients so treated (3). Whether the delayed effect of chelate therapy in Meltzer's patients can be ascribed to the smaller doses of EDTA which were used or whether there is some other unsuspected explanation, the apparent improvement in this second group of patients with angina pectoris is in agreement with the observations of Dr. Boyle's group. It must be emphasized, however, that the value of such therapy cannot be considered proven without a great deal of additional confirmation in a disease with the uncertain natural history of coronary atherosclerosis.

The mechanism of any antiatherosclerotic effect produced by chelating agents is uncertain. At least three suggestions have been advanced. One involves the removal of calcium, possibly from areas where it has been deposited as a late manifestation of atherosclerosis, but probably from situations where it otherwise could be laid down in association with lipid in early atheromatous lesions. It also has been postulated that the parathyroid glands are stimulated by a lowered circulating calcium, causing a more rapid turnover of calcium and thereby affecting atherosclerosis in some way. Finally, it seems possible that the altered availability of a metallic ion, perhaps an alkaline earth or perhaps a transition metal, might affect an enzymatic process or even a physical property, such as membrane permeability, with resultant metabolic effects.

Since calcification occurs late in the development of atheromatous lesions, usually after irreversible anatomic changes have occurred, it seems unlikely that decalcification would be of much clinical benefit. There is, moreover, no convincing evidence that binding calcium significantly limits the deposition of lipid in early atheromatous lesions. That a lowered level of circulating calcium might affect parathyroid or other endocrine glands does not seem unlikely, but there are no data indicating that progression of the atherosclerotic process is thereby delayed. The third postulated mechanism that metabolism is affected by the altered availability of metallic ions is a theoretical possibility which is difficult to demonstrate or exclude.

If and when it can be established that administering the neutral sodium salt of EDTA is beneficial to patients

[1] Established Investigator of the American Heart Association.

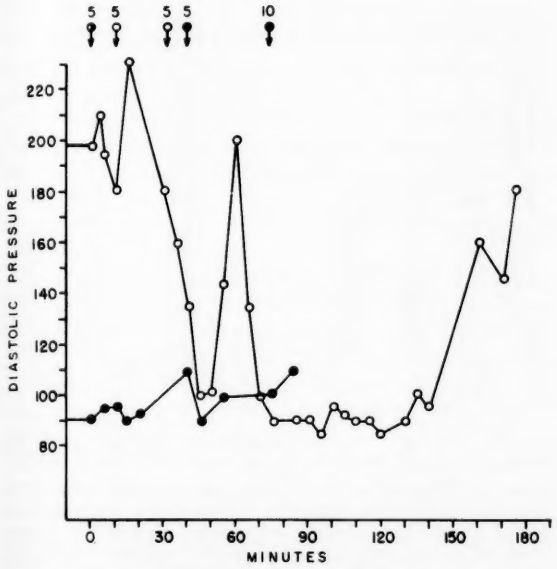


FIG. 1. The antihypertensive effect of $CaNa_2EDTA$ on a pair of anesthetized rats: The open circles indicate diastolic pressure as a function of time for an animal with renal hypertension, and the solid circles indicate diastolic pressure for a normotensive animal. The arrows indicate the times at which the chelate was injected. The numbers above the arrows indicate the size of the dose in milligrams. The character of the circles attached to the arrows (half open and half solid, open or solid) indicates which animals received a particular injection (both animals, the hypertensive animal, or the normotensive animal, respectively). The diastolic pressure of the hypertensive animal was lowered abruptly from very elevated levels to normal by 15 mg of $CaNa_2EDTA$. In contrast, the normal diastolic pressure was not significantly altered by up to 20 mg of the same drug. The reason for the temporary reappearance of hypertension at one and, again, at three hours after the beginning of the experiment is not obvious, but it demonstrates that the effect need not be irreversible or terminal.

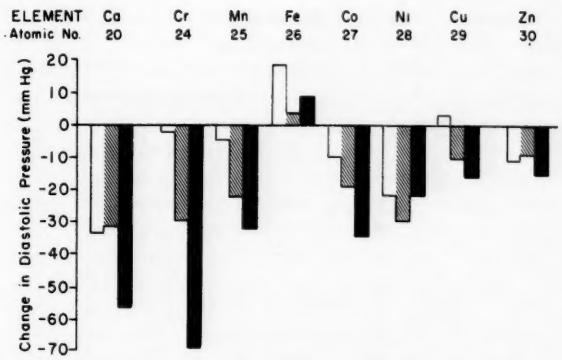


FIG. 2. The antihypertensive effects of divalent EDTA chelates of calcium and zinc and metals of the first transition series. In the figure the transition metals are listed in order of increasing atomic number which is also the order of increasingly tight binding of their divalent chelates. The bars are a measure of the maximum change in diastolic pressure produced by the chelate in question. Specifically, an open bar indicates the average maximum change in diastolic pressure occurring 5 to 20 minutes after the first intravenous injection of 5.0 mg of chelate, a hatched bar indicates the change after a second similar injection, and a solid bar indicates the change after a third similar injection, with the interval between injections approximating 20 minutes. The animals used were rats with renal hypertension; eight were used to assay each metal; and the average diastolic pressure of the entire group of 48 was 120 mm Hg before injection. The comparable antihypertensive effects of the calcium and zinc chelates are cited for purposes of comparison. The antihypertensive effect was maximal for chromium, the most loosely bound of the transition metal chelates. The effect was less marked, but still seemed to be significant for manganese, cobalt, and probably nickel. The effect was insignificant for copper, the most tightly bound chelate. The zinc chelate, with a binding constant approximately equal to that of the nickel chelate, was inert. The potency of the calcium chelate and a possible explanation for the inertness of the iron chelate have been discussed in the text.

with coronary atherosclerosis, it should be possible to determine whether or not the effect is mediated by binding alkaline earth ions. This could be accomplished presumably by determining whether beneficial results persist when the neutral sodium salt is replaced by the calcium disodium chelate which does not bind calcium or magnesium but which does bind transition metals.

Dr. Henry Schroeder and I have had occasion to administer the calcium disodium chelate of EDTA to atherosclerotic patients, not in order to test this point but for entirely different reasons which I should like to outline briefly. Our interest in transition metals originally was aroused by several pharmacologically similar antihypertensive agents for which the mechanism of action was unknown. These agents included thiocyanate, azide, polyphosphates, mercaptans, nitroprusside, and hydralazine. Their only obvious common characteristic was an ability to bind transition metals. This list of antihypertensive agents was not very extensive and a great many pharmacologically active substances are capable of binding metals; none the less we were curious as to whether the antihypertensive effect of these compounds might be related to their ability to bind metal. We therefore decided to test the antihypertensive effect of another powerful metal-binding agent, preferably one which seemed unlikely to have pharmacological effects separate from its ability to bind metals. EDTA seemed to be an ideal compound for our purposes; it was a powerful chelating agent of undertermined antihypertensive potency and it was known to be excreted without being metabolized. Because any alteration in the concentration of circulating alkali metals or alkaline earths might be expected to produce confusing effects on blood pressure, we administered $CaNa_2EDTA$. As is shown in the first figure, the intravenous injection of 15 mg to rats with experimentally induced renal hypertension produced very striking decreases in diastolic pressure. Although the results were not always so marked, significant effects were observed consistently in many similar experiments not only with $CaNa_2EDTA$ but with other chelating agents, such as 8-hydroxyquinoline sulfonic acid (7).

Success in lowering elevated diastolic pressure was followed by an attempt to learn something about what

TABLE 1. *Effect of Parenteral EDTA* on Renal Excretion of Metal*

Day	EDTA* gm IV	Cholesterol mg/100 ml	Urinary Concentration					
			Zn ppb	Mn ppb	Cd ppb	Pb ppb	V ppb	Ni ppb
−2		455	270	5	1	41	19	15
−1		460	220	3	1	9	17	13
0		466	240	4	2	27	17	18
1	1	391	3200	25	1	175	12	16
2	3	307	2800	38	8	205	23	17
3	3	338	2000	29	28	105	29	17
4	3	308	3700	39	43	500	14	16
5	3	314	1700	20	22	34	22	15
6	3	372	1350	14	33	26	25	16
7	3	341	2950	28	37	60	10	20
8	3	349	2150	73	34	68	17	17
9		322	800	5	41	77	13	14
10		314	430	1	29	11	12	14

The table presents data pertaining to one hypercholesterolemic patient. The daily doses of intravenously injected $CaNa_2EDTA$ in grams, the levels of circulating cholesterol in milligrams per 100 milliliters of plasma, and the concentrations of six metals in γ per liter of urine (parts per billion) are cited. The decrease in cholesterol and the increase in all of the metals except vanadium and nickel are obvious.

* As calcium disodium chelate.

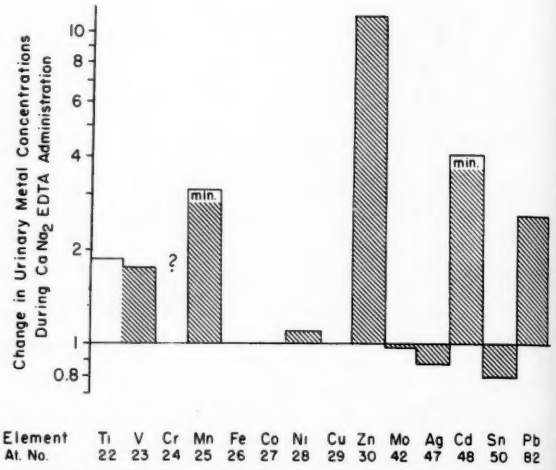


FIG. 3. Changes in urinary metal patterns produced by parenteral $CaNa_2EDTA$. By comparing the urine of the same patients before and after administrating intravenous $CaNa_2EDTA$, changes produced in the concentrations of metals were determined. The data pertain to five patients; all had hypercholesterolemia associated with clinical evidence of atherosclerosis, and none was known to have had an unusual exposure to any metal. The dose of $CaNa_2EDTA$ was 3 gm and it was administered by intravenous drip over a period of three hours. A minimum of three daily 24 hour urines were collected before any chelate was administered, and at least four daily 24 hour urines were collected during its administration. There was less than a 20% increase in urine volume during chelate administration. The metals assayed were transition and related ones. We have not measured the renal excretion of cobalt in any patients, and the urinary concentrations of iron and copper were not determined for these patients. The more than tenfold increase in zincuria seemed unequivocal. A real effect on cadmium and manganese may well have been present, but partially obscured by some control observations which were too low to quantitate; the abbreviation "min." at the top of the bars in question indicates that the plotted increases for these two metals are really minimum values. The approximate doubling of vanadium and lead excretion was less certain. Changes in the concentration of nickel, molybdenum, silver, and tin were not significant. The open bar indicates the considerable uncertainty with respect to titanium concentrations, which were apparently doubled following chelate administration but for which the assay technique was only approximate. The change in chromium concentration seemed to be similar, but the even greater analytic uncertainty for that element is indicated by a "question mark" instead of a bar.

metal might be involved in the effect. It was hypothesized that increasingly tightly bound metal chelates might prove to be decreasingly effective as antihypertensive agents. If they did, it was hoped that the strength of the metal-chelate bond associated with any such change in antihypertensive potency might approximate the strength of the metal-chelate bond involved in the antihypertensive effect. At the same time, it was recognized that in a complicated living system composed of competing chelates, some of which are relatively specific, an equivocal or even an erroneous impression might be obtained. The chelates of divalent ions of the first transition series of elements from $CrNa_2EDTA$ to $CuNa_2EDTA$ were characterized, indeed, by decreasing antihypertensive efficacy, as is shown in Figure 2. Since the supposed ferrous chelate stood overnight in an aqueous solution, its failure to be antihypertensive was ascribed to conversion to the much more tightly bound ferric chelate. A more detailed presentation of these data, including such controls as the effect of free metal ions themselves, has been made (7).

Administration of $CaNa_2EDTA$ to hypertensive patients was obviously the next step. It proved disappointing. Although some antihypertensive responses were observed, they were inconstant and small. It occurred to us that renal hypertension in rats might respond differently from primarily neurogenic human hypertension; but it is still entirely conjectural as to whether a difference in the type of hypertension explains the observed difference in the response of the two species. Although blood pressures were not markedly altered by the parenteral administration of $CaNa_2EDTA$ to patients, their levels of circulating cholesterol were observed to decrease markedly. The effect was consistent, it occurred abruptly, and after discontinuation of the chelate there was a rapid return to the pretreatment level. The decrease in cholesterol was more striking in hypercholesterolemic than in normal patients; a typical example is presented in the Table.

The problem of what metal might be mobilized by the chelate was approached by measuring the renal excretion of transition and related metals before, during, and after parenteral administration of $CaNa_2EDTA$. Except for cobalt, some information was obtained about EDTA-induced changes in urinary concentration for each element in the first transition series from titanium

(atomic number 22) to copper (atomic number 29) and for zinc, molybdenum, silver, cadmium, tin, and lead. The results, which have been presented in detail elsewhere (5, 6), are summarized in Figure 3. A tenfold increase in zinc excretion was characteristic, but no consistent comparable effect was observed for any other metal. Yesterday, Dr. Westerfeld speculated that the explanation for this difference might be found in the unique availability to EDTA of circulating extracellular zinc.

Although the administration of $CaNa_2EDTA$ produced no constant, considerable change in the renal excretion of any metal except zinc, marked increases in urinary concentrations of other metals sometimes occurred. This is demonstrated in the table where the excretion pattern of a woman with coronary atherosclerosis is presented. Manganese, cadmium, and lead, as well as zinc, were significantly altered while vanadium and nickel were not. The return of lead excretion toward normal during the latter part of chelate therapy raises the possibility of exhaustion of some fraction of the stored lead.

Unfortunately, the effect of intravenously administered $CaNa_2EDTA$ is too transient to permit the practical maintenance of lowered levels of circulating cholesterol for protracted periods during which the effects on atherosclerosis could be observed. Unfortunately, too, absorption from the gastrointestinal tract is ordinarily inadequate to produce significant decreases in circulating cholesterol, although some increase in zincuria is often produced (4). Rarely, effective amounts of $CaNa_2EDTA$ are absorbed orally. We have observed a pair of patients with primary xanthomatosis whose levels of circulating cholesterol decreased markedly; moreover, the xanthomatous lesions of one of them regressed and in some areas completely disappeared during three years of therapy (4).

A possible explanation as to how chelating agents affect cholesterol metabolism has been suggested by Drs. Curran and Azarnoff with their *in vitro* observation that several transition trace metals affect cholesterol synthesis (1) and by their *in vivo* demonstration that vanadium can lower circulating human cholesterol (2)

The present situation with regard to EDTA and human atherosclerosis might be summarized as follows:

1) Two independent groups of workers have felt that patients with coronary atherosclerosis were benefited by the neutral disodium salt of EDTA given in large daily parenteral doses for long periods.

2) Although the mechanism of any such effect is unknown, it is known that lipid metabolism can be affected in man by at least one transition metal.

3) In doses similar to the reportedly effective ones of the neutral disodium salt, the calcium disodium chelate of EDTA can lower the level of circulating human cholesterol, presumably by some mechanism other than binding alkaline earths.

4) No effect of $CaNa_2EDTA$ on atheromatous lesions has been observed, and there is no good evidence that affecting cholesterol in any way, or doing anything else that we can do at present, slows the rate of progression of human atherosclerosis.

REFERENCES

1. Curran, G. L. *J. Biol. Chem.* 210: 765, 1954.
2. Curran, G. L., D. L. Azarnoff and R. E. Bolinger. *J. Clin. Invest.* 38: 1251, 1959.
3. Meltzer, L. E., M. E. Ural and J. R. Kitchell. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 132.
4. Perry, H. M., Jr. and G. H. Camel. In: *Metal-Binding in Medicine*, edited by M. J. Seven. Philadelphia: Lippincott, 1960, p. 209.
5. Perry, H. M., Jr. and E. F. Perry. *J. Clin. Invest.* 38: 1452, 1959.
6. Perry, H. M., Jr. and H. A. Schroeder. *Am. J. Med.* 22: 168, 1957.
7. Schroeder, H. A. and H. M. Perry, Jr. *J. Lab. & Clin. Med.* 46: 416, 1955.

Summary remarks by the chairman

HARRY FOREMAN

One cannot help but be impressed by the wide variety of apparently unrelated disorders in which chelating agents have been tried clinically. In certain disorders, such as metal poisoning, the rationale for their use is readily apparent. In others, therapeutic trials have been largely empirical.

Of the many chelating agents that have had clinical trial, EDTA, DTPA, BAL, penicillamine, and dithiocarb have been used with a certain measure of success and have become fairly well established in the treatment of lead, arsenic, copper, chromium, manganese, thorium, uranium, and nickel poisoning. It must be emphasized, however, that although these drugs have been found useful, they are by no means immediately life saving in acute situations, and at present are far from an ideal form of therapy. One hopes that we are just beginning in an era of chelation therapy, rather than at a mature and developed stage.

In the treatment of radioactive metal poisoning, the same situation obtains. The drugs have been found to accelerate the excretion of certain radioactive metals, namely plutonium, uranium, and the rare earths, but there still has not been enough time elapsed to evaluate the effects ot these drugs on the well-being of radioactively poisoned individuals. There is no evidence that chelating agents are useful in the most pressing of the radioactive metal problems, namely radium and strontium.

Of great interest are the unpredicted successes of chelating agents in the treatment of porphyrias. Relief from very distressing symptoms following the administration of chelating agents (EDTA and BAL) has been reported by a number of investigators, who are apparently satisfied that the remissions would not have occurred without the administration of these drugs. The mechanism by which relief is afforded is unknown, but the effectiveness of these agents in ameliorating signs and symptoms suggests that in some fashion trace metals are involved in the biochemical disturbance that is porphyria.

Although there are already many reports of the beneficial use of chelating agents, particularly EDTA, in cardiovascular and sclerosing disorders, one suspects that it will be necessary for the accumulation of much more experience before these results will be unequivocally accepted. When calcium is directly involved, as in digitalis toxicity or in scleroderma, the effectiveness of sodium EDTA is understandable, but in such situations as angina pectoris, hypercholesterolemia, and rheumatoid arthritis, the beneficial value of these drugs is very confusing, and much more study is required.

Summation

MELVIN CALVIN
University of California, Berkeley, California

THE PROGRAM as it is printed bears my name, as you undoubtedly have noticed, and I have been commissioned to give a summation. Now, some of you who know me may have recognized that this meeting has been for me an unusual experience. I have heard every single paper including many of them that weren't on the program, and there is something about each of them in this little green book. I suppose some of you have noticed this.

In thinking over what I could say in summation, I realized that it would be quite hopeless for me to try to leaf through the little book, page by page, and paper by paper and make comments about each of them or even about any of them specifically. I have felt that it would be better for me to try to give you an over-all impression of how the meeting looked to me from the outside because I haven't been inside yet, that is certain.

First of all, I would like to speak for all of us who have participated in this meeting and on their behalf express our heart-felt thanks to Professor Hastings, Dr. Weisiger and the various section chairmen, Drs. Martell, Cotzias, Chenoweth and Foreman who undertook to arrange this program. You will note, as I have forcibly been made to note, that this meeting has brought together men from both ends of the scientific spectrum—the biologists and clinicians at one end, the physical, organic and inorganic chemists at the other end, if you like, with the biochemists and biophysicists in the middle.

On this theme, I thought it would be worthwhile to review my impression of what has appeared on each of these three different levels because to try and translate them, specifically, one into the other, is a bit difficult even though it should be done in specific instances. First of all, I am really overwhelmed by the fact (I don't know whether it is a fact, but the reports were made—how factual the results are or what the implications of the results are is another matter) that the use of material whose primary chemical property is to reduce the thermodynamic activity of a variety of metal ions has had any clinical success at all in the animal organism in most cases. That you have had the temerity to try it, in the present state of our chemical and biochemical knowledge, is to me, in itself, evidence of two things—sheer bravery and the enormous pressures that we have to find ways of handling these problems when we have to resort to using materials whose only claim to action is that they reduce the thermodynamic activity of a whole series of metal ions in solution. That is really all that we know about them.

The fact that there is any clinical success, even vestigial clinical success, is impressive and makes me feel that the biochemists could derive encouragement from this to seek an understanding of what these binding agents are doing. In order to seek an understanding of what the binding agents are doing on the biochemical level, obviously, they must know what the metals are doing. This is basic and, in order to know this, we clearly have to understand the nature of the combination of enzyme with metal or cell membrane with metal and the factors that could influence a change in structure. I will come to those things in a little more detail in a moment. All I want to illustrate here is that the biologist observing nutritional deficiencies or excesses or the clinician observing a disease apparently associated with a metal stimulates the biochemist. The biochemist with his investigation of the effects of metals on enzyme activity, which has been his primary contribution to this business so far, in turn stimulates the biologists and the clinicians and gives them a little moral support if nothing else.

This sort of thing and this sort of meeting provides, I think, the opportunity for these three great, normally separated areas of scientists to make at least some contact with each other and to realize that there may be some mutual interest between them. I know that I have profited a great deal from both sides so that I feel that it cannot help but have profited a certain number of you from whatever side you come. Let us examine each of these three levels. As I have said, the biologists and the clinicians obtain their leads in terms of the metal from their observation of nutritional excesses or deficiencies. Incidentally, there is one remark that I would like to make specifically to Dr. Boyle. He said, "Well, there are a lot of other things that are more active than metals." I agree with this. There certainly are, but the purpose of this conference is to find out where the metals are important and what they can do. This is no denial of the other aspects of biochemistry and physiology, by any means.

The stimulation which the clinicians and the biologists get from their observations of whole organisms or tissues, as the case may be, feeds back and stimulates the chemists. Now let's go to the other end, the physical and inorganic chemist. His primary interest is, or at least until recently was, the electronic structure of the metals and the nature of the interactions of the metals with their environment in trying to describe both the equilibriums and the rates of such interactions. Some of the physical and inorganic chemists and the organic chemists have come from both sides and realize that there may be

something special about the product of such interaction, namely, that, in general, the compounds which are formed between metal ions and the type of groups which we have heard so much about in the last four days actually do have or might have some kind of special properties, catalytic or structural. This has stimulated the physical and inorganic chemists, at least, to make an interface, a contact with the biochemists. This is really where that contact has been made, not so much directly with the biologists and the clinicians, although I think this would be a good idea, too. Perhaps this meeting will bring this about somewhat more prolifically than in the past.

Now, what general areas of investigation are missing? This is the kind of thing I have been asked to try and describe to you. What do I think is missing or, at least, what do I get an impression is missing? Well, one can name a few specific areas. I only heard one mention, in the course of the discussions, of a possibility of metals interacting with nucleotides and nucleic acids, for example. There surely must be a large area there for the biochemists and the physical and inorganic chemists, as well. The investigation of both of these areas has just barely begun. There is another area of investigation which certainly ought to be extended and about which we heard only a few complaints at the beginning of the meeting. Most of our information about the nature of the structure of the molecules that are formed between metals and the various binding agents has been thermodynamic equilibrium constants and, as such, is subject to all of the vicissitudes of such measurements. You heard some of the physical chemists complaining about this. Thermodynamic measurements do not tell us exactly what is happening between the metal and the binding agent and some means should be found to investigate this. Such means are usually spectroscopic methods and there is already a beginning of this in terms of the new microwave spectroscopic methods that are being devised. You heard a little about this and I think that much more will come. We will learn a great deal more of the way in which the metal and the binding agent interact on the electronic level and see what changes in properties of the metal or binding agents may be introduced.

Actually, another area is coming now to the interface between the biochemist, the biologist and the clinician; this, in fact, is an interface that crosses all three areas. That metals are important in biological function I don't think anyone here will dispute. Obviously, that is why we are all here. I am not speaking now of the large amounts of metals which are involved in structure, like calcium, but only of the metals that are present in relatively small amounts. I think this is another differentiation which we should keep in mind. The discussions this morning on sclerotic disease had to do with the deposition of massive amounts of materials, materials which are involved in the structural aspects of the organism rather than in the intimate details of regulation of the mechanism. Up until now I think for most of us, and this has been an overwhelming impression that came out of this meeting, the principal idea was that metals, particularly these trace elements, are somehow involved in the function of enzymes. You heard several reviews of this. There is no doubt that some metals are involved in the function of enzymes. This is not something new, of course. There is a great deal of literature on activation of enzymes by metals. There was some discussion here of what this means and this is a very fundamental discussion. The question of what the metal actually does to the enzyme is basic.

Most of the discussion was based on the notion that the metal has some intrinsic catalytic power either for group transfer (that is, withdrawing electrons and breaking bonds such as magnesium and the manganese effect in the pyrophosphate linkage) or for oxidation-reduction (such as copper, iron and the transition elements generally effect and manganese might effect—which is quite different from its function in the group transfer reaction). I give you these as illustrations of what I know to be underlying the thinking of most of the physical and inorganic chemists and the organic chemists who are working in this area. The biochemists also tend toward this point of view.

However, there is another aspect of metal function only hinted at in the course of the meeting which may very well be as important, or perhaps more so, which I think deserves further investigation. This is the effect of the polyvalent elements, particularly the transition elements, on the secondary and tertiary structure of macromolecules. In the discussion, for example, of the effect of zinc on the activation of carboxypeptidase, alcohol dehydrogenase and several other enzymes, there was some question raised as to whether or not the zinc itself was involved in hydrolysis of the peptide linkage (carboxypeptidase) and whether the zinc itself was directly concerned with the hydrogen transfer reaction (alcohol dehydrogenase). The fact that zinc might be binding at some other point on the macromolecule, changing its shape so as to make the critical site more or less active, as the case may be, was barely suggested. I think this idea should be further explored. There is physical reason to be quite certain that such things happen. For example, just recently we were examining some solutions of ionic polymers to determine their physical behavior in salt solutions and we found that the addition of copper or iron to such polymeric solutions markedly changed the shape of the polymer in solution as evidenced by various physical measurements. Viscosity changes were enormous and, if one makes the change in the right concentration range, one can actually produce a phase change there. What was a homogeneous solution becomes a heterogeneous one; the polymer drops out as a coacervate droplet with all of the iron in it—a very sticky, stringy thing separated from the solution. Therefore, it is quite clear that there are such effects, and there should be such effects, very, very profound changes in the structure of the macromolecule. Now, why do I bring this up? I bring it up, first, because it is something that really hasn't been explored by the

biochemists or by the biophysical chemists as a means of understanding the mechanism of action of the metals, either as activators or as deactivators, as the case may be.

Even more important, it seems to me, is the possibility that such phenomena may play a role in the passage of materials through biological barriers of various kinds. An actual change of the structure of the barrier may be induced by the presence of trace amounts of metals of suitable type and oxidation number (they could be very specific just as they are for the enzymes). I can visualize physical-chemical ways in which such barriers might be profoundly influenced and this really hasn't been examined much either from the physical-chemical point of view or from the biochemical point of view. There has been much discussion of this subject by the biologists and the clinicians; and they recognize, as do the pharmacologists, that this is a major problem in understanding the mechanism of action either of inhibitors or activators. The possibility that the surrounding of the metal ion by a binder will have an effect on this kind of function has appeared time and time again in the course of the clinical and biological discussions, but there is relatively little really good knowledge of how this might happen from the biochemical or the physical-chemical point of view. That it can happen, that it must happen, I think every physical chemist will agree. But how does it happen, when does it happen, to what extent does it happen, and what are the conditions under which it happens? This they cannot define unequivocally.

What is on the third side? What is missing among the clinicians and the biologists? Well, I know it is presumptuous of me to try to tell them what is missing; but there seems to be a great deal that I would like to know and I am sure that you would like to know. You heard a little of this already this morning in a request from a biochemist for more information about tissue analysis. Well, this obviously is something which the biologists and the clinicians can do, and only they can do. They are the only ones who have the materials available. In order to understand what a metal is doing, either activating or deactivating, inhibiting or accelerating a chemical reaction or a transition process, we must know where it is. I was very much stimulated by the kind of information that came from the knowledge that there is some relationship between vanadium and chromium in cholesterol synthesis. This is fine at that level. That is only just the beginning from my point of view. I think from the point of view of the biochemists, also, it is only just the beginning. It is a lead which the clinician provides. What we need in more detail from the clinician is the location of the vanadium in the organism. Where is the manganese in the organism, where is the bound manganese, and what are the changes in the tissue distributions? What is the relation between the addition of vanadium to the tissue and the distribution of manganese in the tissue? These are the kinds of information which the biochemist would like to have in order to know where to start looking for enzymes, if there are enzymes, or for interfaces, or for membranes, which might be affected by such metals. As I say, it is hard for me to prescribe where the biologist should look except to mention that, obviously, there are all sorts of metals. There are sixty or so metals and probably most of them are in the organism. The question is where are they and in what form. This can then be transmitted to the biochemists who will try to find out something about what these metals are doing. I would encourage the continued effort to try and find biological and clinical effects of these various types of structures as they are brought forth at the whole organism, microorganism or tissue level. There is, for example, Albert's discovery, in one instance, of the apparent necessity to have both a binding agent and a metal to have a biological effect and the binding agent is certainly not a natural one. I don't have any clear concept of why this works. This is something it seems to me on which the biochemist and the cellular physiologist should work together. The physical chemist also should cooperate because, clearly, in order to understand this problem it is going to require some fundamental and very intimate knowledge of metal and binder interaction, solubility relationships and things of this type. I don't know exactly in which direction to look, but this is the kind of collaboration which needs extension without any doubt.

I am thinking now at a narrower and narrower level and beginning to pick up particular things, which I really didn't want to do. I wanted to mention only the areas which I felt were missing. To skip back a little bit to the physical chemists and the biochemists, only one really good model for the catalytic effect of metal was presented at this meeting, namely, the one by Snell and Metzler on transamination. There certainly are others, for example, transphosphorylation with lanthanum as a catalyst, established some years ago; there are various others which should be explored further both by the physical and inorganic chemists and by the biochemists so that the detailed mechanism of the action of each of these metals will provide some more understanding in their enzymatic function.

I think perhaps it is worth saying, from this general point of view, that I feel very much encouraged by the bravery of the clinicians. I am a little discouraged by the speed, or lack of it I should say, with which the physical or inorganic chemists are moving into biology, but they are moving. Some biochemists and some biophysicists have been in the middle for some time and their numbers are growing at a great rate. I think that they are drawing from both sides and that we can look forward to stimulation from the biologists and clinicians, on the one hand, from the physical and inorganic chemists, on the other, and from the biochemists and biophysicists in the center. With this I would say that I hope that in another year or two, at another such gathering, there will be more representatives from the physical sciences here than there are today. There are only half a dozen or so. There should be more from the physical side. I hope that we will be able to speak each other's language a little more easily then.

Each time we meet, we should have a greater ease in communication.

Let me show you one of the unexpected, completely unexpected, profits of this meeting. I heard Dr. Philips describe the eye structure of the cat, which has zinc in it. This was entirely new to me. I had never heard of such a thing and I questioned him about it a bit later. After some discussion, and, of course, I haven't had time to get a hold of a cat, yet, as Dr. Philips suggested, it appears unlikely that the zinc compound functions as a mirror. I think it is more likely to be a device for converting infra-red quanta into visible quanta. We have a physical analogy for this. This would be a more reasonable thing, it seems to me, for the cat to do than what had been so far suggested. When I get home, I'll go into this and see whether these things can happen. At the same time, I think that the biologists will use this idea, also, and will find out how far into the infra-red the cat can see. I don't know, maybe that is already known, but we can't solve that problem today. Thank you very much.

CLOSING REMARKS

DR. FOREMAN: Thank you very much, Dr. Calvin. Dr. Hastings, do you want to say anything before we adjourn?

DR. HASTINGS: Since I began this meeting as a student, I wish to tell you, my faculty, that I've gotten a great deal out of this conference. I want to thank all of you, my teachers, my fellow students of whom there are some here, I am sure, the chairmen of the sessions, the speakers, and especially that great chelator, Dr. Calvin. I had concern for a time that this conference was becoming somewhat schizophrenic, but I find that, if it was, we found a cure. It was not EDTA but MCTA—Melvin Calvin, Teacher Atomic.

Thank you all for coming. The conference is adjourned.

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